Maternal control of seed development mediated by the flavonoid biosynthesis pathway

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Maternal control of seed development mediated by the flavonoid biosynthesis pathway

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Dedicated to:

My parents

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Abstract

Many plants exhibit post-zygotic barriers to hybridisation and failure of crosses is frequently associated with abnormal endosperm development. The "triploid block" is one such example that results from intraspecific crosses between diploid and tetraploid plants. Here, paternal excess usually leads to severe endosperm over-proliferation, failure of cellularisation and consequent arrest of embryo development. Most ecotypes of *Arabidopsis thaliana* tolerate 2xX4x crosses which result in enlarged viable seeds, however the Columbia (Col-0) ecotype exhibits a classic triploid block. Previous work suggested that some genes involved in regulation of the flavonoid biosynthesis pathway (FBP) could act as maternal modifiers of the Col4x-mediated triploid block, re-imposing a normal (balanced) developmental programme on the endosperm. The FBP operates in a specialised maternal layer of the seed coat, the endothelium, which lies adjacent to the endosperm.

This study set out to understand the role of the FBP and its products in the regulation of seed development following 2xX4x crosses. To this end, a collection of FBP mutants was assembled to test their effect on alleviating the triploid block. Both light and confocal microscopy was utilised to assess the effects of FBP mutations on seed development and biochemical analysis (ESI–LC–MS/MS) was used to determine if particular products of the FBP were significant. In addition, a potential link between the hormone auxin and the FBP was investigated in seeds by utilising FBP mutants carrying the fluorescent auxin "response" reporter DR5rev::GFP. This work revealed that many (though not all) mutations of the FBP lead to alleviation of the triploid block and that, in particular, perturbations to the pathway that result in a reduction of proanthocyanidins or affect normal vacuolar biogenesis in the endothelium are associated with 'rescue' from Col4x-induced seed lethality. Preliminary evidence is presented that does suggest a potential link between auxin transport and products of the FBP though how this may operate is unclear.

Abbreviations & Acronyms

Bp	Base pair/s
Col -0	Columbia ecotype (Col 2x)
Col 4x	Tetraploid in Col-0 ecotype
CZE	Chalazal endosperm
DAP	Days after pollination
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EMS	Ethyl methanesulfonate
F1	First filial generation
F2	Second filial generation
Kb	Kilo base pairs
KD	Knock down
KO	Knock out
KV	Kilovolts
LC-ESI-QQQ-MS/MS	Liquid chromatography electrospray ionization triple quadruple mass spectrometry
L*er* 2x	Diploid in Landsberg *erecta* ecotype
Min	Minutes
mRNA	Messenger RNA
Mseconds	Milliseconds
NASC	Nottingham Arabidopsis Stock Centre
PCR	Polymerase chain reaction
PE	Peripheral endosperm
Psig	Pounds per square inch gage
RC	Regenerated Cellulose
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase-PCR
S.E.	Standard error
Sec	Seconds
TAE	Tris-acetate-EDTA
Ws 2x	Wassilewskija-2x
WT	Wild type

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Chapter 1: General Introduction

1.1. *Arabidopsis thaliana* as a model organism

A. thaliana is a small flowering plant native to northern Europe that is widely used as a model organism in plant biology. *A. thaliana* is a member of the mustard family (Brassicaceae), which includes cultivated species such as cabbage (*Brassica oleracea*), oilseed rape (*Brassica napus*) and radish (*Brassica sativus)* (Somerville and Koornneef, 2002).

A. thaliana is widely utilised as a convenient model plant due to its rapid life cycle of about six weeks from germination to seed maturation, prolific seed production and its easy cultivation in restricted spaces. The *A. thaliana* genome has several advantages for molecular studies because it is small, consisting of ~130 MB and containing ~ 25,000 genes. This is however not the case in crop species with large genomes such as *Triticum aestivum* (~16000 MB) and *Brassica napus* (~1200 MB), and molecular studies are more difficult with these species (Arumuganathan and Earle, 1991). Extensive genetic and physical maps exist of all five chromosomes and *A. thaliana* genes are easy to manipulate via transformation using *Agrobacterium tumefaciens* and ethyl methanesulfonate (EMS) (a chemical mutagen) for efficient mutagenisis. The TAIR website (www.arabidopsis.org) provides extensive information about genes, markers, polymorphisms, maps, clones, proteins and gene families. Furthermore, there are seed stock centres, such as the Nottingham Arabidopsis Stock Centre (NASC), which hold collections of naturally occurring ecotypes (Garcia-Hernandez and Reiser, 2006). NASC also holds a wide variety of T-DNA insertion mutants (Alonso *et al.*, 2003), which enables the identification of the roles played by genes with a hitherto unknown function.

1.2. The importance of seed size

The global population is projected to increase to around 9.1 billion by 2050 and, therefore, the need to increase food production is of great importance (FAO, 2009). Even at current population levels (~7 billion), approximately 850 million people are undernourished, many are unable to feed their families and are suffering from chronic malnutrition (FAO and IFAD, 2012). Seeds constitute about 68% of the world's food supply and are thus a vital source of nutrition (Li and Berger, 2012). The monocot cereal

endosperm, a product of a second fertilisation event in angiosperm reproduction, is a particularly important source of nutrition and calorific content and it is estimated that this tissue accounts for approximately 60% of calories consumed (Berger, 2003). Moreover, in monocots, most of the mature seed's volume and weight is taken up by the endosperm. However, not all production is for direct consumption by the human population and much is used as a raw material in the production of animal feed and biofuel (Sabelli and Larkins, 2009). In dicot species, for example *A. thaliana*, the final seed weight also depends upon the proliferation of the endosperm (Baud *et al.*, 2008), though, at seed maturity only a relatively small amount of this tissue remains in the form of a thin layer adjacent to the seed coat (Baud *et al.*, 2002).

Thus, as seed development progresses in *A. thaliana* the highly proliferated endosperm, which is the primary store of nutrients, is reduced as a result of the growth of the embryo (Hirner *et al.*, 1998; Baud *et al.*, 2008). Therefore, the endosperm plays a role in regulating the flow of nutrients to the developing embryo (Lopes and Larkins, 1993).

Importantly, the increasing use of crops for liquid biofuel production, i.e. ethanol and biodiesel, could have consequences such as increased crop demand and decreased food availability. It is, therefore, vital that we find a way to improve agricultural output to meet the demands for food production and for other sectors such as the biofuel industry. In order to do this, we need to develop further our understanding of seed development (Figure 1.1) (FAO, 2009; Li and Berger, 2012).

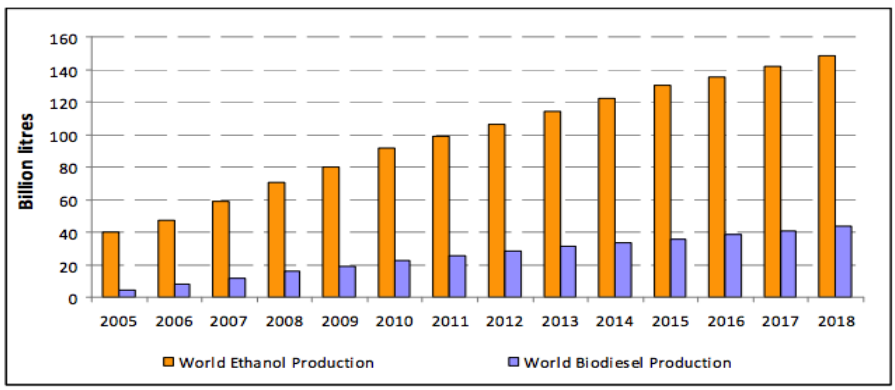


Figure 1. 1: Global ethanol and biodiesel production projected to 2018 (adapted from FAO, 2009).

Plants have been artificially selected and bred for increased yield in agriculture for millennia and most food grains consumed today have seeds significantly larger than their wild relatives (Sundaresan, 2005). It is now understood that endosperm plays a pivotal role in the regulation of seed size (Scott *et al.*, 1998) and is thus a potential target for research aimed at improving overall crop yield. In addition, endosperm development that proceeds following double fertilisation represents a potential barrier to hybridisation and thus gaining an understanding of the mechanisms that regulate its development could facilitate plant-breeding programmes (Bushell *et al.*, 2003).

1.3. Seed development in angiosperms with a focus on *A. thaliana*

The plant life cycle involves alternation of generations between the multicellular haploid (gametophytic) and a diploid (sporophytic) phases with gametes being produced by male and female gametophytes (Sundaresan and Alandete-Saez, 2010). Sexual reproduction in plants involves double fertilisation (see Figure 1.2.C) and the resulting seeds are made up of three components; the embryo, the endosperm and the seed coat (testa), all of which are genetically different from one another (Figure 1.2.D) (Gehring *et al.*, 2004). The development of seed coat is determined by the maternal genome, while the endosperm and the embryo develop under the control of both parental genomes (Li and Berger, 2012). There are two distinct phases in seed development: embryo development and seed maturation (Park and Harada, 2008).

Most flowering plants, including *A. thaliana*, have an embryo sac containing seven cells, which consist of four different cell types. This is classed as the Polygonum-type of embryo sac (Figure 1.2.A and B). The four different cell types are as follows: the egg cell, two synergid cells (which are significant for pollen tube attraction), the central cell and three cells called antipodal cells that have currently unknown functions. The whole embryo sac is surrounded by diploid integument tissues and together these constitute the ovule. The integuments will develop to form the seed coat in the mature seed (Sundaresan and Alandete-Saez, 2010; Gehring *et al.*, 2004). The ovary, the space that houses the ovules, and the integuments are maternally derived.

The egg cell (n) is fertilised by one of the two sperm cells (n) delivered via the pollen tube, resulting in a diploid zygote (embryo 2n) which grows long and then splits asymmetrically, producing a smaller apical cell and a larger basal cell that develop into an embryo and its suspensor, respectively (Raz *et al.*, 2001; Baud *et al.*, 2008; Park and

Harada, 2008) (Figure 1.2.C and D). The central cell (2n) is fertilised by the second sperm cell (n) giving rise to the multinucleated triploid endosperm, which functions as a nutrient source for the embryo, which would be in the pre-globular stage, after which endosperm cellularisation initiates (Figure 1.2.C and D). The ovule integuments differentiate to form the seed coat (testa) which will protect the seed during embryogenesis, dormancy and germination (Sundaresan and Alandete-Saez, 2010; Gehring *et al.*, 2004; Haughn and Chaudhury, 2005; Doughty *et al.*, 2014).

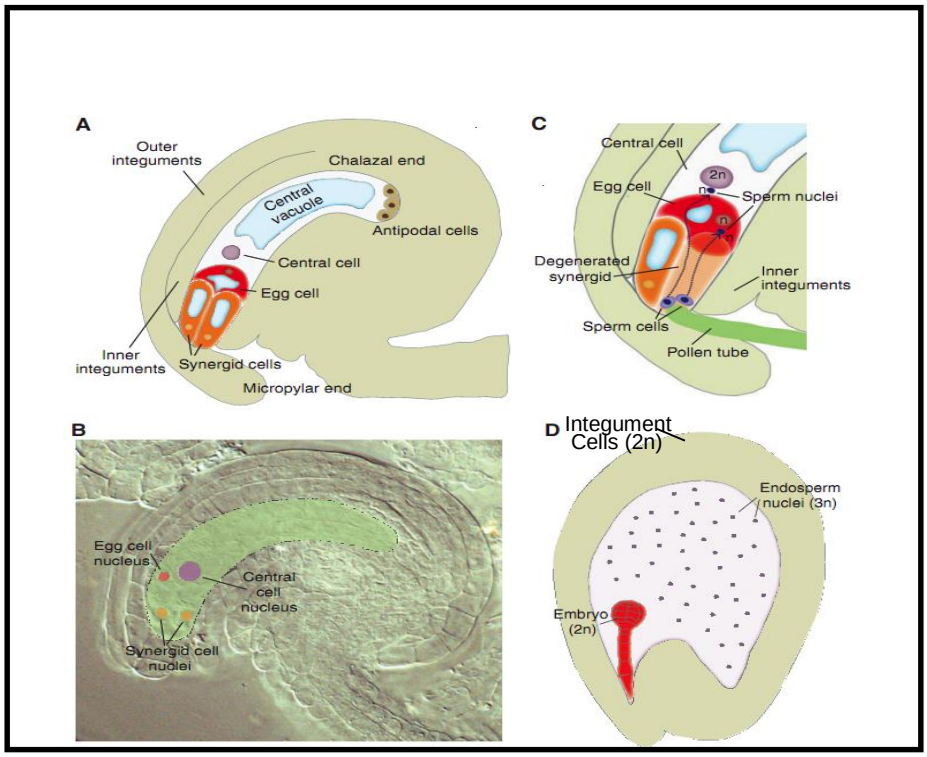


Figure 1.2: The female gametophyte, double fertilisation and early seed development. A. Diagram of the female gametophyte (embryo sac) with four cell types: the synergid cells (orange), the egg cell (red), the central cell (purple) and the antipodal cells. **B**. The ovule of wild-type *A. thaliana*. **C**. The diagram displays the mechanism of double fertilisation. **D**. Sketch of a developing seed before endosperm cellularisation, with an embryo (2n) at the globular stage and endosperm nuclei (3n) (adapted from Sundaresan and Alandete-Saez, 2010).

1.3.1. The development of integument layers

In higher plants the seed coat has a number of crucial functions and thus understanding its structure and role in seed development has been an important goal for biologists. In the *A. thaliana* seed, the embryo and endosperm are surrounded by five cell layers of maternal tissue that make up the seed coat, comprised of two outer and three inner cell layers (Figure 1.3). The seed coat functions to protect the embryo against physical damage, pathogen attack and from damage by ultraviolet light. In addition, the seed coat controls germination by maintaining the embryo in a dehydrated dormant state (Windsor *et al.*, 2000). Moreover, the morphology of the seed coat has been used in the past as a useful taxonomic trait (Moise *et al.*, 2005). Interestingly, the seed coat also plays an important role in the coordinated regulation of seed development which is an essential feature of this project (see section 1.7). Thus, the significance and role of each layer of the seed coat will be described at this point. The layers will be discussed running from outer to inner: the epidermal layer (oi2), the palisade (oi1), third layer (ii2), fourth layer (ii1') and the endothelium (ii1), respectively (Figure 1.3).

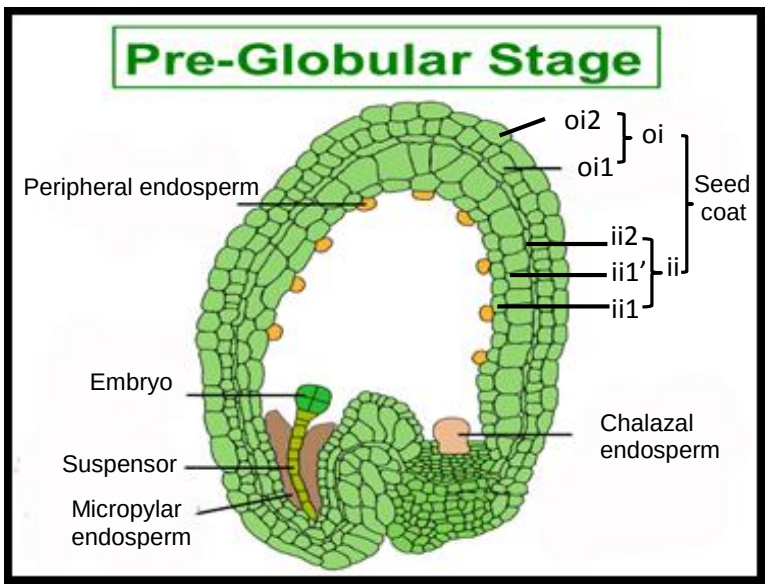


Figure 1.3: Shows two outer and three inner integument layers in *A. thaliana* seed. The outer integument (**oi**) includes: the outer epidermis of outer integument layer (**oi2**) and inner epidermis of outer integument layer (**oi1**) termed 'palisade'. The inner integument (**ii**) layers are composed of outer layer of inner integument layer (**ii2**), a median layer of inner integument layer (**ii1'**) and pigment layer is also called 'endothelium' (**ii1**). Beeckman *et al.'s* (2000) labels for the integument layers are used (adapted from Hashimoto, 2013).

The outer integument is formed by two cell layers, the epidermal and palisade layers. The outer, epidermal, layer is formed by a single layer of cells that have a large central

vacuole; this layer produces mucilage in later stages of seed development (Beeckman *et al.*, 2000; Windsor *et al.*, 2000). At the globular stage of embryo development, an accumulation of starch granules forms both near the outer cell wall of the outer epidermal layer and the inner cell wall of the inner palisade layer (Beeckman *et al.*, 2000; Windsor *et al.*, 2000). After fertilisation, the size of both the epidermal cells and their central vacuoles increase immediately. This process eventually leads to the formation of a columella (columnar shape) inside the cytoplasm. At the same time, the grain starch begins to shrink away, as does the vacuole. The starch granules are used to form those polysaccharides required to strengthen cell walls (Moise *et al.*, 2005). After the mucilage (a pectic polysaccharide) has been secreted and the columella has been formed, a secondary cell wall forms outside the columella (Bradford *et al.*, 2007). At late stages of maturation, the seed coat is dehydrated then absorbed, followed by the extrusion of mucilage around the entire seed. Various studies have indicated that mucilage plays an important role in successful seed germination, particularly in arid conditions (Penfield *et al.*, 2001; Bradford *et al.*, 2007).

Mutations that affect outer integument development in *A. thaliana* frequently result in a reduction or altered composition of mucilage (Haughn and Western, 2012) (Table 1.1).

Table 1.1: Example of a gene that has an effect on outer integument development in *A. thaliana* seeds.

Gene	Function	References
YABBY family transcription factor INNER NO OUTER (INO)	Regulator of outer integument differentiation	Radchuk and Borisjuk, 2014

The palisade layer that lies under the epidermal layer of the outer integument, may consist of one or more cell layers and is typically parenchymatous, though it is sometimes collenchymatous, or sclerotic. By the late stages of seed development this layer is largely collapsed (Beeckman *et al.*, 2000). Initially, starch granules in the palisade enlarge and then at torpedo stage, begin to shrink, whilst the central vacuole divides into two or three smaller vacuoles. Thinner tangential and radial walls are often characteristically thickened in this layer. As already mentioned, starch granules in the epidermal layer usually contribute to the strengthening of the cell wall and also the palisade layer.

Having outlined the characteristics of the two outer integuments, the three inner layers of the *A. thaliana* coat will be described. The cells of the third (ii2) and fourth (ii1') layers of the inner integument are highly vacuolated, in particular on the abaxial side of the seed

(Beeckman *et al.*, 2000). At the walking-stick embryo stage, as the embryo grows, these layers begin to shrink and disintegrate (Kuang *et al.*, 1995; Beeckman *et al.*, 2000). When maturity is reached, the two layers of the inner integument do not differentiate anymore and are crushed together (Haughn and Chaudhury, 2005). The fifth cell layer (the endothelium (ii1)) is called the pigment layer because of the accumulation of pigments within the vacuole of this layer. The cells of the endothelium are small cuboid parenchyma isodiametric cells containing a few grains of starch, dense cytoplasm and small vacuoles (resembling meristematic cells), which are compressed at maturity (Beeckman *et al.*, 2000; Moise *et al.*, 2005). During the early stages of development (the two-cell embryo stage), the endothelium layer is filled with light yellow pigments. Most of these pigments are proanthocyanidin (PA) flavonoid compounds that accumulate in the central vacuole, and are synthesised during the early stages of development - around 1–2 days after pollination (DAP). From the micropylar region, the pigments progress toward the chalaza until around 5–6 DAP (note, for an overview of seed structural features and development see figure 1.3). These pigments are later oxidised, giving the seed coat a brown colour (Beeckman *et al.*, 2000; Moise *et al.*, 2005; Lepiniec *et al.*, 2006; Bradford *et al.*, 2007; Radchuk and Borisjuk, 2014). The genes and transcription factors needed for the synthesis and compartmentation of the PA flavonoid compounds have now been identified and classified (Haughn and Chaudhury, 2005). The comprehensive transcriptomic analysis of testa development in *A. thaliana* testa has been useful in understanding the process in other species (Radchuk and Borisjuk, 2014).

The pathway responsible for flavonoid biosynthesis in seeds appears to be generally conserved between species. For example other members of the Brassicaceae such as oilseed rape (*Brassica napus*), *Brassica rapa* and *B. oleracea*, have been found to contain orthologs of BANYULS (Auger *et al.*, 2009), which encodes the anthocyanidin reductase gene *ANR* (Albert *et al.*, 1997). However many domesticated legumes and cereals have lost the genes which underlie PA synthesis and this commonly results in pale seeds that have hindered understanding of the regulation of this pathway in these species. Thus, the cultivated form of barley (in common with wheat and rice) does not amass significant amounts of PA compared to the wild form (Radchuk and Borisjuk, 2014). Several research groups are investigating the relationship of secondary PA metabolism with both the developmental regulation and the stress response as this has the potential to contribute significantly to the improvement of crops in the future (Radchuk and Borisjuk, 2014).

In the early stages of seed development pigments fill the vacuole of the endothelium and also accumulate in the cytoplasm in the later stages. Accumulation of pigment is at its maximum in the early torpedo embryo stage of development at around 6-7 DAP. In the late torpedo stage, the pigment disappears from the centre of the cells and remains only at the periphery. At maturity, the endothelium layer breaks down, so that only the dead cells still exist; and because of this it is impossible to distinguish them from the cell layers above (Beeckman *et al.*, 2000; Moise *et al.*, 2005). As reported by Debeaujon *et al.* (2000), there is an accumulation of flavonoids in all the three cell layers (layers ii2, ii1' and ii1) of the inner integument. In mature seeds, around 15 DAP, the palisade and three inner cell layers (ii2, ii1' and ii1) that make up the seed coat are desiccated, dead and have been crushed together. The remaining endosperm at this stage is pushed outwards against the inner integument and forms an aleurone layer. The epidermis, on the other hand, maintains its shape by virtue of the thick secondary cell wall of the columella (Haughn and Chaudhury, 2005; Moise *et al.*, 2005). Ultimately all five cell layers die and their death occurs in a specific sequence. This suggests that cell death is programmed as part of the differentiation process. The outer integument layers - the epidermis and palisade- undergo cell death first, followed by that of the inner integument (Haughn and Chaudhury, 2005). The death of the integument cell layers is likely to trigger the release of PAs that become oxidised, giving the brown colour of the seed coat.

Moise *et al.* (2005) suggested that the seed coat weakens and ruptures during germination and this contributes to generating components that contribute to biotic- and abiotic-stress resistance. Recently, numerous studies have attempted to show that the seed coat (maternal tissue) is essential in directing the nutrient supply towards the embryo during early seed development (Locascio *et al.*, 2014; Radchunk and Borisjunk, 2014). Thus, the correct establishment of the seed coat and the endosperm are essential prerequisites for appropriate embryo development.

As the testa is semi-permeable, it does not block gaseous exchange (Welbaum and Bradford, 1990). The epidermis of mature seeds has few or more usually no functional stomata (Geisler and Sack, 2002). This suggests a limited capacity for gas exchange, particularly when the chemical composition of the cuticle is taken into consideration (Sinclair, 1988). Most gas exchange activity in pea seeds happens in the micropylar region (Wager, 1974). Synchroton X-ray computer tomography has allowed to see air spaces in developing *A. thaliana* seeds. Intercellular spaces that are gas-filled are

probably necessary for the movement of oxygen within the seed. Carbon dioxide diffuses through plant tissue more easily than oxygen, as (unlike oxygen) carbon dioxide can dissolve easily in water and thus, in the form of carbonate ion, can move from cell to cell (Radchuk and Borisjuk, 2014).

1.3.2. The development of endosperm

1.3.2.1 Genomic structure of the endosperm

As outlined in (section 1.3) the endosperm is the second fertilisation product in flowering plants. The fertilisation of the egg cell (1 maternal; 1m) by the haploid sperm cell (1 paternal; 1p) results in a diploid zygote (2n; having a 1m: 1p genome ratio). However, the second haploid sperm cell (1 paternal; 1p) fuses with the central cell (2 maternal; 2m), creating the triploid (3n) endosperm, which functions as a nutrient source, provided by the seed parent, for the developing embryo during germination or embryogenesis (Bushell *et al.,* 2003; Gehring *et al.,* 2004; Haughn and Chaudhury, 2005; Sundaresan and Alandete-Saez, 2010; Li and Berger, 2012). Hence, the endosperm and the embryo, in most species, would both have the same alleles; however, the endosperm (3n) would have an extra maternally derived genome (Scott *et al.*, 1998).

1.3.2.2 Endosperm development

The endosperm in *A. thaliana* seeds develops in two steps during embryo morphogenesis; proliferation and cellularisation. Proliferation is the initial period of endosperm development and happens a few days after fertilisation, where nuclei replicate freely in the endosperm. In *A. thaliana*, a minimum of 16 nuclei is produced in the endosperm before the zygote divides for the first time (Sabelli and Larkins, 2009). The endosperm grows rapidly which contributes to the overall increase in seed size. Multinucleate endosperm coenocytes (multiple nuclei in the same cytoplasm of a single cell) are formed as a result of proliferation due to nuclear divisions not being followed by cytokinesis (Figure 1.2 D above). Coenocytic endosperm can have up to several thousand nuclei in some species though more typically ~ 200 in *A. thaliana* and up to 512 in maize (Olsen, 2001; Olsen, 2004; Berger *et al.*, 2006; Li and Berger, 2012).

Endosperm cellularisation of the coenocytes occurs by the formation of a cell wall around the nuclei (Figure 1.4), starting after the eighth mitotic cycle in *A. thaliana*, and is completed by the torpedo stage of embryo development. The endosperm is then consumed, acting as an energy source for the embryo as the seed develops (Lafon-Placette and Kohler, 2014; Yan *et al.*, 2014). Cellularisation acts to stop further increases in the volume of the seed. Endosperm cellularisation initiates at the micropylar region, and moves inward towards the central chamber of the embryo sac to the chalazal pole (Brown *et al.*, 1999; Olsen, 2001; Berger *et al.*, 2006; Li and Berger, 2012).

Endosperm development is classically 'bipolar' and results in micropylar and chalazal regions (Kranz and Kumlehn, 1999; Berger, 2003; Olsen, 2004; Berger *et al.*, 2006). Moreover, and in *A. thaliana*, the endosperm develops into three distinct regions during seed maturation. The embryo surrounding region is micropylar endosperm (MCE), the central peripheral endosperm (CPE) which forms a lining around the wall of the developing embryo sac, and the chalazal endosperm (CZE) which contains the chalazal endosperm cyst (CEC), which forms adjacently to the vascular connection with maternal tissue so that the endosperm and/or the embryo can receive maternal nutrients (Olsen, 2004). The chalazal region remains syncytial and it continues to form nodules until the latter stages of seed maturity (Brown *et al.*, 1999).

During the last period of embryo development, much of the endosperm is crushed and absorbed by the expanding embryo (Brown *et al.*, 1999; Beeckman *et al.*, 2000; Olsen, 2001; Berger *et al.*, 2006).

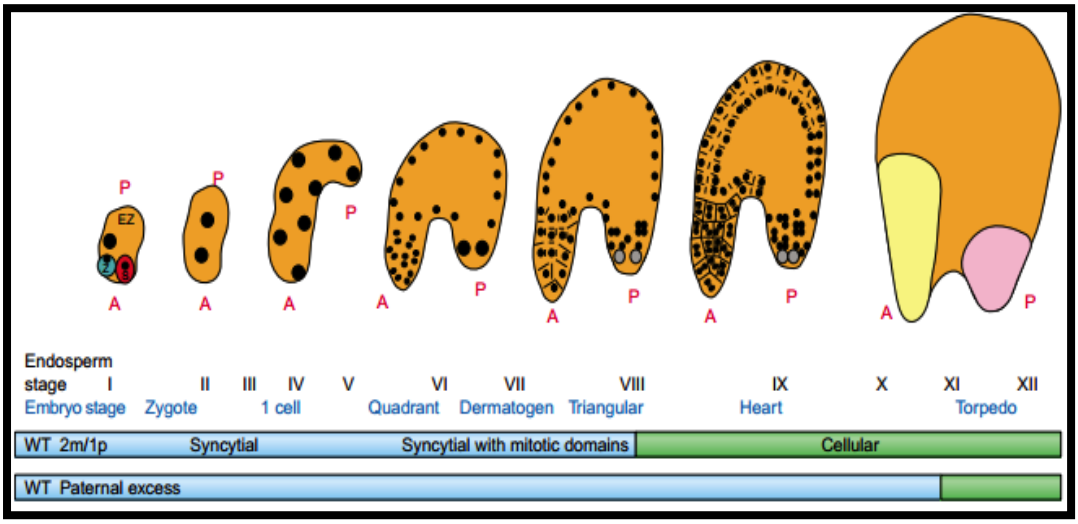


Figure 1.4: Endosperm development in *A. thaliana*. When double fertilisation of the embryo sac occurs the egg cell (**Z**) produces the zygote and the diploid central cell (**EZ**) produces the endosperm. Endosperm development proceeds in two main phases- a proliferation phase followed by endosperm cellularisation. Developmental stages are marked by successions of pseudo- synchronous mitoses. There are three mitotic areas, from the anterior pole (**A**) to the posterior pole (**P**). Areas are micropylar endosperm (yellow), peripheral endosperm (orange) and chalazal endosperm (pink). After the eighth mitotic cycle, the peripheral endosperm is cellularised. The blue bar represents the proliferation phase and the green bar represents the cellularisation phase. **Abbr.**: **S**: synergid; **WT**: wild-type (adapted from Berger, 2003).

1.3.2.2.1. Nodule development from chalazal pole

The micropylar pole induces differentiation of the endosperm leading to endosperm cellularisation. The pole containing the chalaza endosperm cyst induces proliferation of a multinucleate endosperm (syncytium) leading to nodule formation. The nodules are cysts that develop in the peripheral endosperm. The size and number of the nodules are affected by endosperm proliferation (Figure 1.4 and Figure 1.5) (Scott *et al.*, 1998; Brown *et al.*, 1999; Tiwari *et al.*, 2010).

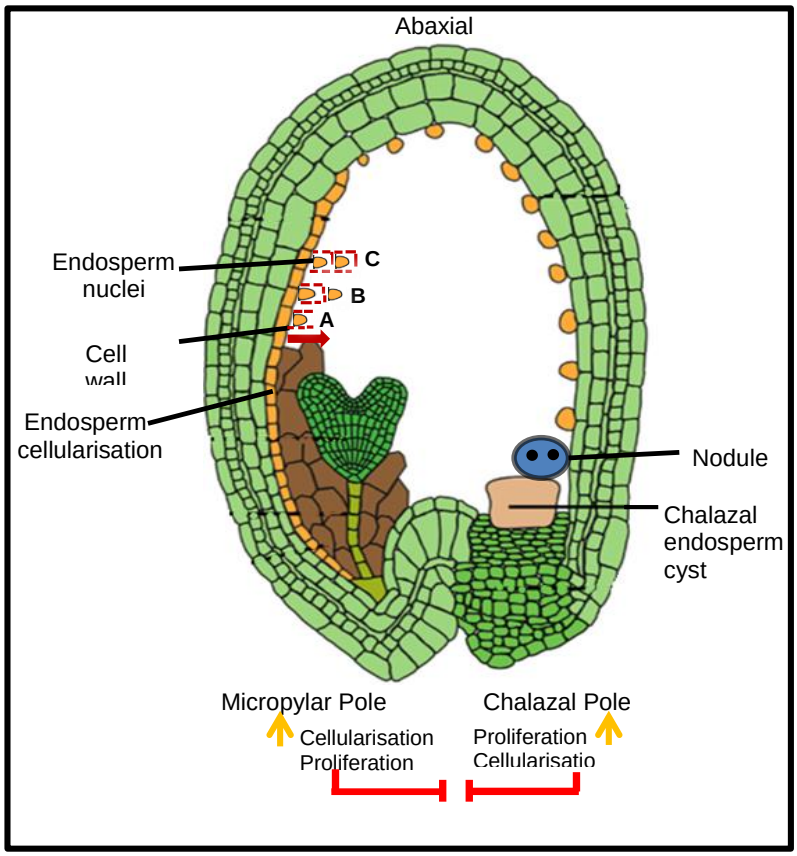


Figure 1.5: Control of endosperm cellularisation. At the early/ heart stage of embryo development, endosperm cellularisation occurs quickly. Cellularisation starts from the micropylar pole and moves gradually through the central cell to the chalazal pole, then moves inward towards the central chamber of the embryo sac. Initiation of cellularisation corresponds to the beginning of cotyledon development in the embryo. **A**, **B** and **C** show the formation of cell walls around each nucleus, which starts from the edges and moves into the central chamber. The model shows two poles: the micropylar pole and chalazal pole. Both poles have an inhibitory effect on each other. The orange arrows indicate the process being favoured by each pole, whereas the red line indicates the process being inhibited by the poles. The micropylar pole activates endosperm cellularisation early and inhibits endosperm proliferation and vice versa for the chalazal pole.
For seeds having significant paternal excess in the endosperm (e.g. 2xX4x cross), cellularisation generally occurs at the chalazal pole much later and proliferation of the chalazal pole occurs significantly earlier, resulting in a phenotype with more nodules (adapted from Hashimoto, 2013).

1.3.2.3 The role of the endosperm in seed development

A. thaliana, like most other dicotyledons, degrades its endosperm as the seed matures and in doing so provides space for the embryo to develop. The evidence suggests the role of the endosperm is as a transient nutrient storage tissue (protein, carbohydrates and oil) prior to delivery of these resources to the embryo (Hirner *et al.*, 1998; Li and Berger, 2012). Once endosperm cellularisation is completed in *A. thaliana*, no further proliferation occurs (Ungru *et al.*, 2008). This is in contrast to monocot crops such as wheat or barley where the endosperm is a major storage area of nutrients and persists until maturation (Locascio *et al.*, 2014).

As well as being a vital source of nutrition for both animals and humans, the endosperm is also crucial to the survival of the next generation of the plant (Focks and Benning, 1998). Furthermore, in being pivotal to embryo development, final seed size and content are indirectly dependent on the endosperm (Noguero *et al.*, 2015).

The embryo, endosperm and integuments need to synchronise their growth during the various stages of seed development. Studies have suggested that the interaction between the endosperm and integuments during growth is an important factor in the determination of seed size (Garcia *et al.*, 2003; Garcia *et al.*, 2005; Luo *et al.*, 2005). Moreover, a correlation between final seed weight and the amount of endosperm proliferation has been experimentally observed (Schruff *et al.*, 2006). Findings from studies by Sorensen *et al.* (2002) suggest that *A. thaliana* mutants that are defective in endosperm cellularisation have small seeds at maturity because of inadequate nutrients flow to the embryo.

1.3.2.4 Endosperm development and its molecular regulators

It has been established that seed size is significantly affected by the development of the endosperm (Sabelli and Larkins, 2009) and thus a large body of research has concentrated on the identification of genes that regulate development of the endosperm (Kohler *et al.*, 2010; Kohler and Makarevich, 2006).

Following interploidy crosses endosperm development is frequently perturbed and it is likely that this results from dysregulation of the *FERTILISATION-INDEPENDENT* SEED (*FIS*) target genes, as the *FIS* complex loses control of endosperm development (Erilova *et al.*, 2009). The findings of Scott *et al.* (1998) and Dilkes *et al.* (2008) show that the seed phenotype of interploidy crosses that deliver paternal excess are characterised by endosperm over-proliferation and late endosperm cellularisation which often results in the arrest of embryo development and then the collapse of the seed. Indeed, it is established that normal embryo development requires proper development of the endosperm (Berger *et al.*, 2006).

Mutations that influence endosperm development (Portereiko *et al.*, 2006; Bemer *et al.*, 2008), cellularisation (Pignocchi *et al.*, 2009) or breakdown (Waters *et al.*, 2013a) can affect, interrupt or even stop embryo development (Locascio *et al.*, 2014). If the endosperm fails to develop or cellularise, this has an effect on the amount of nutrients

essential for embryo maturation that are stored in the seed, such as sucrose (Ruan *et al.*, 2010; Locascio *et al.*, 2014).

1.3.2.5 Endosperm development in maize: Maternal effect vs. Paternal effect

Paternally Expressed Genes (PEGs) and Maternally Expressed Genes (MEGs) that are associated with particular stages of endosperm development (Raissig *et al.*, 2013). The maize, *MEG1* imprinted gene is involved in the flow of nutrients from maternal tissues to the seed and as a consequence has a role in controlling the size of the seed (Costa *et al.*, 2012). Transfer cell specification and development is positively controlled by Meg1, and the absorption of nutrients is thus augmented. Its influence can be clearly seen at 10 DAP, when the period of maximum auxin levels in the basal endosperm transfer layer (BETL), the embryo-surrounding region (ESR) and aleurone layer coincides with the beginning of the accumulation of starch and storage protein (Lur and Setter, 1993; Sabelli and Larkins, 2009; Locascio *et al.*, 2014). Some genes that are imprinted are involved in hormone signalling pathways. In this respect, auxin plays a critical role in correct seed development in accordance with genetic imprinting (Xin *et al.*, 2013).

The part played by imprinted genes, *MEGs* in particular, in determining the right nutrient synthesis is as significant as the mediation of transport by auxin. Unsurprisingly, the auxin transport protein PIN1 was discovered as specific *MEG* (Xin *et al.*, 2013). It was also demonstrated that YUC10 orthologs in *A. thaliana,* rice and maize are exclusively expressed paternally, and this suggests that the function of YUC10 may be central and fundamental to endosperm development (Waters *et al.*, 2013b; Locascio *et al.*, 2014). At this stage of development, the role of PEGs and MEGs is to enable the co-ordination and communication between an embryo, endosperm and maternal tissues (Locascio *et al.*, 2014).

1.3.2.6 Embryo development and the impact of developmental transitions of the endosperm

Embryogenesis (embryo development) in higher plants consists of two major phases – morphogenesis and maturation (Olsen, 2001; Berger *et al.*, 2006; Park and Harada, 2008). Morphogenesis is the process by which the body plan of the embryo is established, whereas maturation involves cell expansion and accumulation of the storage of

macromolecules to provide nutrition and protection in preparation for desiccation, germination and early seedling growth (Park and Harada, 2008). *A. thaliana* seed development takes roughly three weeks from pollination at day 0 under natural light and temperature conditions (Baud *et al.*, 2008).

Moreover, it has been demonstrated that embryo viability is dependent on endosperm cellularisation by the use of mutants which result in endosperm cellularisation failure (Table 1.2), which indicates how important the result of endosperm cellularisation is on the developing embryo.

Table 1.2: Examples of genes that have a crucial role in endosperm cellularisation in *A. thaliana* seeds.

Gene	Function	References
FERTILISATION INDEPENDENT SEED POLYCOMB COMPLEX2 (FIS-PRC2)	As mutant lead to endosperm cellularisation fails, causes seeds to abort with their embryos arresting at the heart stage of development	Chaudhury *et al.*, 1997; Lafon-Placette and Kohler, 2013
ENDOSPERM DEFECTIVE1 (EDE1)	Show cellularisation failure and concomitant embryo arrest in interploidy crosses	Pignocchi *et al.*, 2009; Lafon-Placette and Kohler, 2013
Type I MADS-box transcription factor regulator AGAMOUS LIKE 62 (AGL62)	Negative regulator of endosperm cellularisation	Li and Berger, 2012; Lafon-Placette and Kohler, 2013

The main route for nutrients and growth regulators in the early stages of seed development is the suspensor, which connects the embryo proper with the endosperm and surrounding maternal tissues. Morphogenesis ends when the embryo reaches the heart stage of its development when there is a transition from radial to bilateral symmetry at 6 DAP. At this point, the suspensor degenerates and the embryo proper will take nutrients directly from the endosperm (Lafon-Placette and Kohler, 2014). The maturation phase initiates with a crucial developmental change that is marked by cellularisation of the endosperm (Goldberg *et al.*, 1994). From this point, the embryo obtains sucrose directly from the endosperm rather than relying on the suspensor to transport nutrients.

The large central vacuole, which is the seed's most significant storage area for hexoses, shrinks as a result of endosperm cellularisation. In early seed development, the central vacuole quickly converts the imported sucrose into hexoses, thus determining sink strength. This process is probably facilitated by invertase situated in the vacuole. As endosperm cellularisation progresses, the dimensions of the central vacuole diminish and this, in turn, results in a reduction in the ratio of hexoses to sucrose. The reduction of

central vacuole sink strength is thought to be necessary to allow the embryo to become the seed's main sink. It is only after endosperm cellularisation that fast embryo growth and the accumulation of storage product takes place. Because of this, failure of endosperm cellularisation will probably result in the embryo not receiving enough sucrose; as this has not been transported to the central vacuole. From this, we can conclude that successful seed development requires tightly regulated endosperm cellularisation (Lafon-Placette and Kohler, 2014). The maturation phase can be divided into three stages. The first phase occurs around 7-10 DAP, when the embryo develops to fill the whole seed (Raz *et al.,* 2001). The ratio of the embryo to endosperm volume is gradually reversed, and the embryo goes through a process of rapid division and expansion while the endosperm is gradually degraded until it comprises just one cell layer surrounding the embryo. Photosynthetic pigments accumulate in the embryo causing the colour to turn green. At the early stages of maturation, embryos have high concentrations of starch, with storage lipids and proteins beginning to accumulate (Baud *et al.,* 2008). The second phase initiates at around 11-16 DAP. At this stage, fatty acid and protein synthesis are increased in the embryo with starch levels falling (Baud *et al.*, 2002). A gradual increase in seed weight occurs throughout this developmental stage, due to these biosynthetic activities. Late maturation occurs at around 17-20 DAP. Storage compound synthesis arrests and the seed becomes metabolically inactive and tolerant to desiccation. To prepare for seed dormancy, the water content of the seed drops dramatically from 32% to 10% (Baud *et al.*, 2002). Seed growth is largely related to the growth undergone by both the endosperm and the integuments during the embryo morphogenesis phase as shown in (Figures 1.3 and 1.6) (Baud *et al.*, 2008).

As the FIS-PRC2 complex negatively controls AGL62 expression, it follows that endosperm cellularisation is under epigenetic control (Lafon-Placette and Kohler, 2014). A consequence of this is that endosperm development can be perturbed if the balance of maternal and paternal genomes is altered as can be the case in interploidy crosses (this will be explained in more detail in section 1.5).

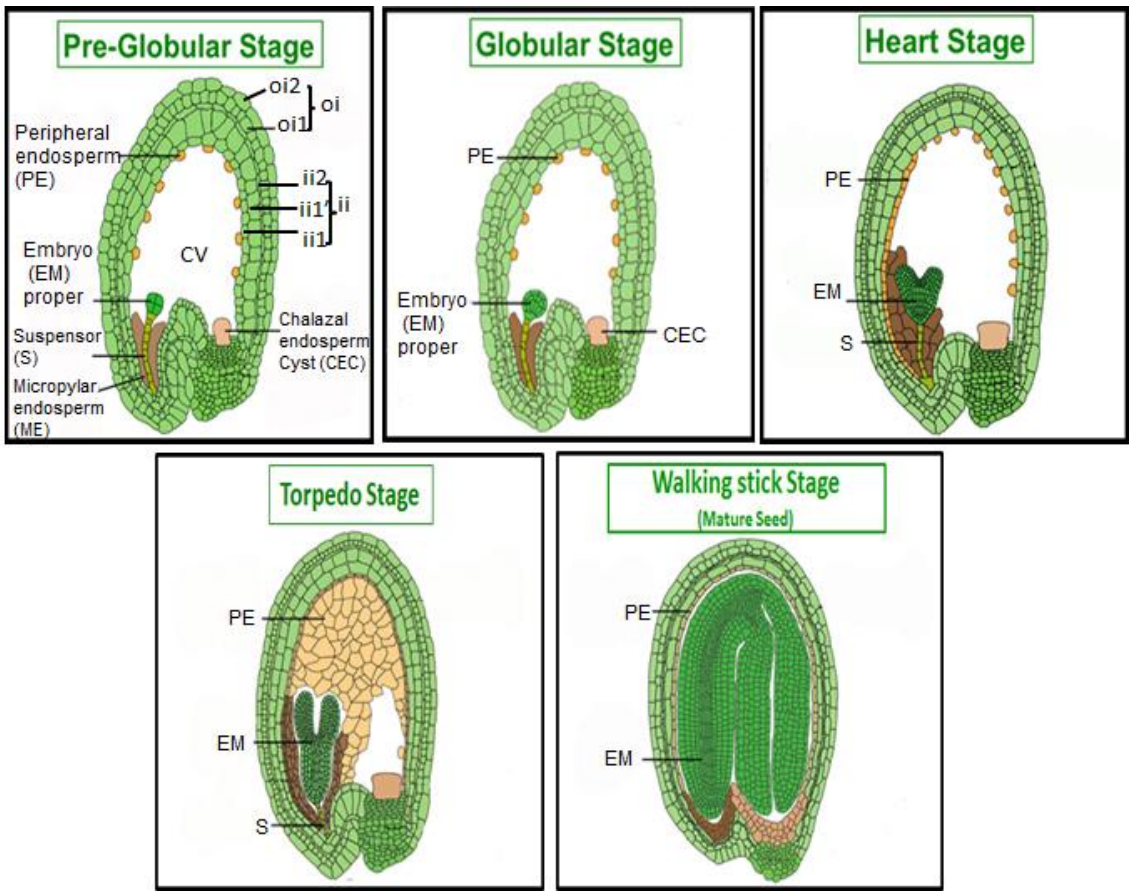


Figure 1.6: The five stages of seed development in *A. thaliana*. Abb.: CEC: chalazal endosperm cyst; **PE:** peripheral endosperm; **CV:** central vacuole; **EM:** embryo; **S:** suspensor; **ME:** micropylar endosperm (adapted from Hashimoto, 2013).

1.4. Hybrids and hybridisation barriers in plants

Plant hybridisation has revolutionised the agricultural domain. Hybrids can be either interspecific or intraspecific. Interspecific hybrids are formed when the parents are of two different species, whilst intraspecific hybrids are formed by crosses of parents of the same species but having different ploidy levels (chromosome numbers) (Rieseberg and Carney, 1998).

Hybrids are rare but can occur naturally in the wild. Hybridisation can produce more disease resistant plants with larger seeds. An example of the utility of hybridisation is the selective breeding of rice, a process that has led to the advancement of crop yield, disease resistance and hardiness in this species. This process has consequently helped to combat world hunger (Breseghello and Coelho, 2013). However, the majority of hybrids fail due to hybridisation barriers (Rieseberg and Carney, 1998).

In fact, hybridisation barriers have evolved to minimise gene flow between different species (Dilkes *et al.*, 2008). They promote speciation and the generation of a greater

diversity of plants. Speciation via polyploidy is common in flowering plants, accounting for roughly 4-10% of all speciation events (Otto and Whitton, 2000). The establishment of intraspecies polyploidy has played a pivotal role in the evolution of multicellular eukaryotic lineages (Rieseberg and Carney, 1998).

There are two main classes of hybridisation barrier; pre-zygotic and post-zygotic (Rieseberg and Carney, 1998). Pre-zygotic barriers arise from the failure of the egg to become fertilised, whilst post-zygotic hybridisation barriers arise from the premature death of the zygote after fertilisation. Pre-zygotic fertilisation barriers can occur by varied mechanisms. For example, failure to effect fertilisation can be caused by the pollen not adhering to the stigma in the flower, abnormal growth of the pollen tube or by differences in the flowering times of the parents (Bushell *et al.*, 2003). Post-zygotic barriers arise after successful fertilisation, for example, due to a genomic imbalance in the embryo causing sterility in the offspring and even embryonic lethality in F1 hybrids (Bushell *et al.*, 2003). One cause of hybrid inviability that is most relevant to this project is the imbalance of imprinted genes in the endosperm that results from interploidy crosses (Bushell *et al.*, 2003). Such an imbalance can lead to failure of seed development as is depicted in figure 1.7.

1.5. The parent-of-origin-effect on the development of the seed- Triploid block

Generally, the normal development of endosperm involves a parental genomic ratio of 2 maternal to 1 paternal (2m: 1P), which is the ideal balance between the paternal proliferation genes (growth promoters) and the maternal growth inhibition genes to form a healthy seed. Any disruption of this balance can cause developmental defects and even lethality (Figure 1.7). A maternal excess (a ratio > 2m: 1p) is usually correlated with inhibited proliferation of the endosperm; a paternal excess is generally correlated with over-proliferation of the endosperm (Bushell *et al.*, 2003).

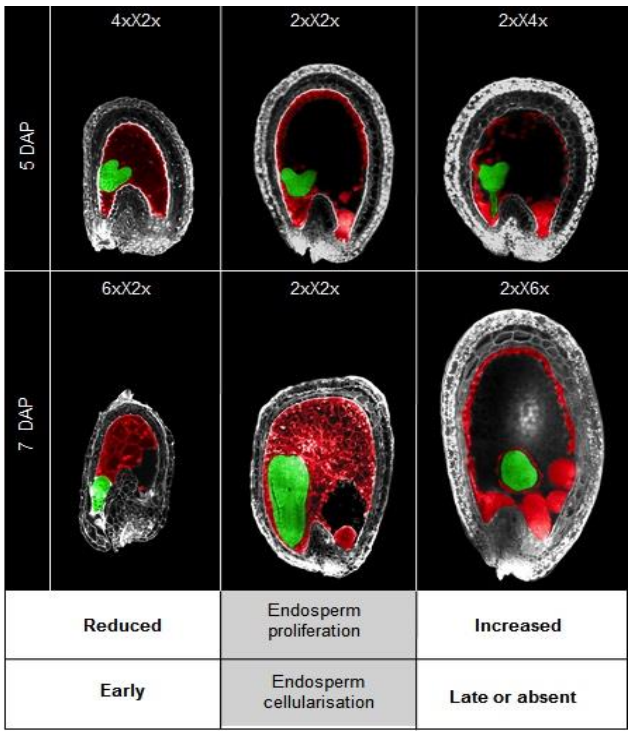


Figure 1.7: Confocal laser scanning photomicrographs of developing seeds in *A. thaliana* at 5 and 7 DAP; show that the ratio of the maternal and paternal contribution in interploidy crosses affects the viability and size of seeds. 2xX2x crosses have the normal paternal genome contribution and produce a normally sized viable seed. 2xX4x crosses have double the normal amount of paternal genomes, which causes the seeds to have accelerated endosperm nuclei mitosis and delayed endosperm cellularisation, resulting viable but abnormally large seeds. However, 4xX2x crosses have doubled the normal amount of maternal genomes and the seeds have reduced endosperm mitosis and precocious cellularisation -this cross results in viable but abnormally small seeds. The seed from 2xX6x and 6xX2x crosses show similar but more extreme phenotypes to 2xX4x and 4xX2x crosses, however, they are observed to produce non-viable seeds (adapted from Scott *et al.*, 2013).

Parental imprinting is when differential gene expression in the offspring is dependent on the parent of origin and it is controlled by epigenetic modifications in the gamete's genome. This mechanism is almost certainly ubiquitous amongst flowering plants and has been extensively documented in *A. thaliana* (Kermicle and Alleman, 1990; Haig and Westoby, 1991; Scott *et al.*, 1998; Li *et al.*, 2013). Thus, alterations in the levels of gene expression would occur if the normal parental genome ratio were altered in some way. To ensure normal seed development, it is important to maintain the 2m: 1p ratio in the endosperm. This is a more important factor than the maintenance of the 1m: 1p embryo ratio (Kermicle and Alleman, 1990; Haig and Westoby, 1991). Viable plant embryos can develop without a paternal contribution (e.g. 1m: 0p), however, they do need maternal and paternal contributions to the endosperm (Scott *et al.*, 1998). An essential component of the mechanism of imprinting is DNA methylation, and this has been seen to be critical

in imprinting in maize (Lund *et al.*, 1995; Finnegan *et al.*, 1998). In interploidy crosses in *A. thaliana*, DNA methylation has also been demonstrated to be a vital part of the parent-of-origin effect. Together these data suggest that methylation is a critical aspect in parental imprinting in flowering plants (Adams *et al.*, 2000). Imprinting will have an effect on the 'perceived' parental genome ratio in the endosperm, causing it to differ from the normal parental genome ratio. Extra doses of maternal genomes would lead to a reduction in growth in the endosperm (due to earlier cellularisation) and, therefore, a smaller seed due to the presence of extra copies of growth inhibitors. Conversely, extra doses of paternal genomes result in an increase in endosperm proliferation and, therefore, larger seeds are formed. The result of this is that endosperm growth is affected by a 'tug-of-war' between the maternal and paternal genomes (Spielman *et al.*, 2001; Bushell *et al.*, 2003).

There are several classic examples of post-zygotic barriers that arise because of the presence of polyploidy e.g. the triploid and tetraploid block (Haig and Westoby, 1991). Polypoidy is thought of as an important speciation mechanism and is very common amongst flowering plant lineages. Polyploids possess a nucleus with more than two sets of chromosomes (Kohler *et al.*, 2010). The triploid block is a common post-zygotic breeding barrier in plants, though *A. thaliana* is unusual in that it typically has a tetraploid block (Scott *et al.*, 2013). A triploid block, named because the resulting embryo is triploid, can be due to mating a diploid maternal parent with a tetraploid paternal parent (2xX4x = 3x triploid embryo) (Figure 1.7). Likewise, a tetraploid block arises when a hybrid embryo carries a tetraploid chromosome number, e.g. from a diploid maternal parent and a hexaploid paternal parent (2xX6x = 4x tetraploid embryo) (Figure 1.7). The results of these crosses give non-viable progeny due to an imbalance of imprinted genes that regulate endosperm development. Excess paternal genomes present in the endosperm following these crosses lead to over-proliferation of this tissue and late cellularisation. In cases where the triploid block is overcome, and progeny is able to grow as sporophytes, they encounter problems during meiosis as an absence of chromosome pairing results in gametes that are typically aneuploidy, having unbalanced chromosome sets or sterility. In spite of this, the triploid block and the difficulties resulting from odd numbered chromosome sets being separated during meiosis can be overcome, as the most frequent method of polyploid formation is likely to be via unreduced gametes and individuals with deviant numbers of chromosomes (Johnston *et al.*, 1980; Johnston and Hanneman, 1982; Kohler *et al.*, 2010). Thus gaining an understanding of post-zygotic hybridisation barriers

is critical if we are to overcome them to potentially generate new useful hybrids and find means to manipulating seed size that could result in yield increases in agronomically important crops.

1.5.1. *A. thaliana* ecotypes differ in endosperm responses to interploidy crosses

There are many different *A. thaliana* ecotypes (over 750) that are widely distributed across the world and each possesses a different tolerance to interploidy crosses (Weigel and Glazebrook, 2002; Bolbol, 2010). The majority of plants (for example Maize**)** exhibit a post-zygotic triploid block. Most ecotypes of *A. thaliana* however are more tolerant to interploidy crosses than other plants (Nishiyam and Inomata, 1966). For example, the C24 and Landsberg *erecta* (L*er*) ecotypes have a higher tolerance to crossing with polyploid parents. These ecotypes do not demonstrate a triploid block in crosses between 2xX4x parents because over-proliferation of the endosperm is more limited in these lines. However, they do exhibit a tetraploid block with 2xX6x parents where a high degree of seed abortion is observed. These ecotypes exhibit an extreme over- or under-proliferation of endosperm that goes with an increase in paternal or maternal contributions respectively; therefore, *A. thaliana* exhibits a 'tetraploid block' as a general feature (Dilkes *et al.*, 2008; Scott *et al.*, 2013). There are, however, ecotypes such as Columbia (Col-0), which exhibits a triploid block.

The *A. thaliana* L*er* and Col-0 ecotypes are commonly used for research. As mentioned above L*er* is tolerant to crosses with parents having higher ploidy, whereas Col-0 is intolerant to such crosses in terms of seed development. This Col-0 intolerance is evident in crosses between diploid (2x) seed parents and tetraploid (4x) pollen parents (Dilkes *et al.,* 2008). These crosses lead to a high degree of seed lethality (75-90%) with over-proliferation of the endosperm being a characteristic feature. Conversely, the reciprocal cross (4xX2x) demonstrates a very low level of lethality accompanied by under-proliferation of the endosperm which produces small seeds like those produced by L*er* and C24 4xX2x crosses (Dilkes *et al.*, 2008). On the other hand, intraploidy (2xX2x and 4xX4x) balanced crosses are observed to have virtually no seed lethality (Bushell *et al.*, 2003). The high levels of abortion induced by Col 4x sperm is similar to that observed in 2x X 6x crosses for most other ecotypes (Scott *et al.*, 1998), suggesting that Col-0 displays an asymmetric hybridisation barrier. As mentioned above hybridisation barriers

in diploid by tetraploid crosses (e.g. a triploid block) are rare in *A. thaliana* (Bushell *et al.,* 2003). Other species that exhibit a triploid block (2xX4x lethality) are not usually asymmetric as is observed in the Col-0 ecotype of *A. thaliana*.

This asymmetric hybridisation barrier resulting from Col4x sperm shows that there is a 'paternal' triploid block. The mechanism by which Col4x sperm induces lethal over-proliferation of the endosperm is currently not understood, however, it would seem that Col4x sperm transmits a lethal 'factor' that results in massive over-proliferation, or loss of control over the proliferation of the endosperm (Dilkes *et al.,* 2008). So, is this sperm lethal factor due to somatic chromosome doubling or due to failure to reduce chromosome number? And does this lethal factor act rapidly or slowly? (Kohler *et al.*, 2010).

1.6. Regulation of seed size – the roles of zygotic and maternal tissues

The mature testa has distinct inner and outer integuments (see section 1.3.1). After double fertilisation, development of the zygotic tissues of the embryo and endosperm influences differentiation of the inner and outer integuments layers to produce the morphology of the mature testa. Likewise, the seed coat itself also influences the development of zygotic tissues immediately after fertilisation (Roszak and Köhler, 2011; Moise *et al.,* 2005). As previously mentioned, defects in mature seeds, abortion or even death can happen as a result of a failure in 'communication' through the structure of these compartments or in their development. Some of the communication pathways between the seed structures have been understood by the characterisation of mutants, which present phenotypes that affect the development of the seed (Locascio *et al.*, 2014).

There are several factors that play a vital role in the seed coat to determine seed size. Firstly, the maternal factors that are known to act in the seed coat to influence the size of the seed include: *TRANSPARENT TESTA GLABRA2 (TTG2), APETALA2 (AP2), AUXIN RESPONSE FACTOR2 (ARF2) / MEGAINTEGUMENTA (MNT), DA1, KLUH/CYTOCHROME P450 78A5(KLU/CYP78A5), DA2* and DNA METHYLTRANSFERASE1 (MET1) (Figure 1.8)(Garcia *et al.*, 2005; Schruff, 2006; Li *et al.*, 2008; Doughty *et al.*, 2014; Locascio *et al.*, 2014).

Several maternal transcription factors act to restrict the growth of the seed size (Table 1.4). In contrast, two of the maternal transcription factors enhance seed size through the regulation of cell expansion *(TTG2)* and the proliferation of cells in the seed coat *(KLU / CYP78A5)*(Figure 1.8) (Garcia *et al.*, 2005; Xia *et al.,* 2013).

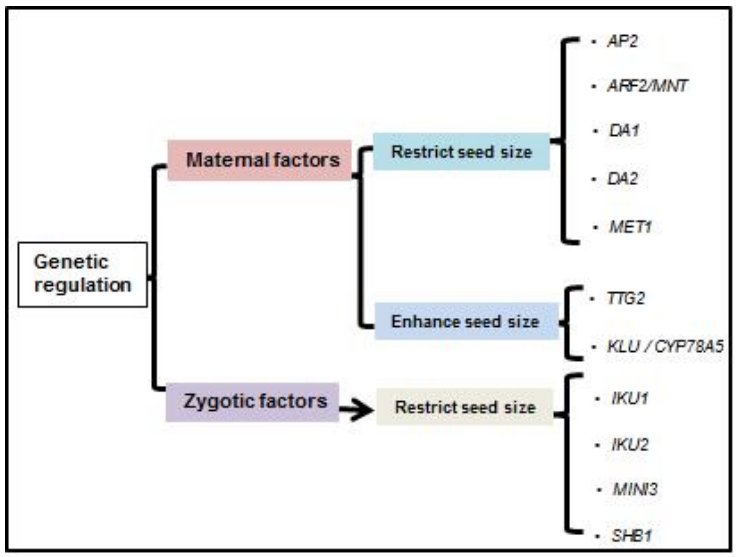


Figure 1.8: A model for genetic regulation of seed size in *A. thaliana* (modified from Garcia *et al.*, 2005).

Secondly, zygotic factors that are central in determining seed size are components of the *HAIKU (IKU)* pathway. *IKU1*, *IKU2*, *MINISEED3 (MINI3)* and *SHORT HYPOCOTYL UNDER BLUE1 (SHB1)* regulate early endosperm development (proliferation) and also expand the seed cavity (Luo *et al.*, 2005; Wang *et al.*, 2010) (Figure 1.8; Table 1.3).

Table 1.3: Examples of genes that restrict the growth of seed size in *A. thaliana*.

	Gene	Gene function	References
Maternal transcription factors	*APETALA2 (AP2)*	Regulates the size of seeds via affects the development of the endosperm and embryo	Jofuku *et al.*, 2005; Ohto *et al.*, 2005; Ohto *et al.*, 2009
	AUXIN RESPONSE FACTOR2 (ARF2) / MEGAINTEGUMENTA (MNT)	Work by stopping cell proliferation in the integuments	Adamski *et al.*, 2009; Xia *et al.*, 2013
	DA1 and *DA2*	Restrict the proliferation of cells in the integuments	Adamski *et al.*, 2009; Xia *et al.*, 2013
	DNA METHYLTRANSFERASE1 (MET1)	Inhibits proliferation and elongation of seed coat integuments	Li and Berger, 2012
Zygotic transcription factors	*HAIKU (IKU)* pathway: *IKU1*, *IKU2*	Triggers seed growth and endosperm proliferation	Garcia *et al.*, 2005; Luo *et al.*, 2005; Wang *et al.*, 2010
	MINISEED3 (MINI3)		Garcia *et al.*, 2005; Luo *et al.*, 2005; Wang *et al.*, 2010
	SHORT HYPOCOTYL UNDER BLUE1 (SHB1)		Locascio *et al.*, 2014

Thirdly, Sun *et al.* (2010) suggest that growth-producing phytohormones (of maternal and zygotic origins) auxins, brassinosteroids (BR), and cytokinins (CKs) are significant signalling molecules in the development of seeds in *A. thaliana*. Moreover, crosstalk

between auxin and Abscisic acid (ABA) pathways plays a part in germination and seed dormancy (Liu *et al.*, 2007; Liu *et al.*, 2013).

1.6.1. *TRANSPARENT TESTA GLABRA2 (TTG2)* and regulation of the Flavonoid Biosynthesis Pathway

The crucial role of the seed coat in regulating the development of the endosperm has been emphasised by the identification of the DR.STRANGELOVE1 (DSL1) locus, a powerful maternal modifier of the Col syndrome. Detailed mapping of the locus identified *TRANSPARENT TESTA GLABRA2 (TTG2)* as the gene responsible for this modifying activity (Dilkes *et al.*, 2008). Seed parents that carry the *ttg2* mutation dramatically rescue seed abortion in 2xX4x crosses in L*er* and -to a lesser degree- in Col-0. This discovery indicates that the flavonoid biosynthesis pathway (FBP) could titre an endosperm cellularisation factor, as TTG2, a transcription factor, is an important positive regulator of the FBP.

TTG2 encodes a transcription factor of the WRKY family expressed weakly in the endosperm and at a high level in the seed integument (Johnson *et al.*, 2002; Garcia *et al.*, 2005). *TTG2* acts within the maternal tissue of the sporophyte, which suggests that there is an interaction between the maternal and seed generations that is responsible for controlling interploidy mating. *TTG2* mutants act maternally to counteract the effect of a paternal excess by forcing endosperm cellularisation (Dilkes *et al.*, 2008) and seeds from mutants are small (Debeaujon *et al.*, 2000). *TTG2* mutants also exhibit reduced cell elongation in the integuments and more precocious endosperm cellularisation than in the wild-type (Garcia *et al.*, 2005). A lack of pigmentation in the seed coat is a characteristic of *ttg2* mutants and the accumulation of proanthocyanidin is reduced in the endothelium when fertilisation occurs (Dilkes *et al.*, 2008). The endothelium is the third layer of the inner integument (see Figure 1.6 ii1). *ttg2* also has an effect on the production of mucilage and development of the trichome.

There are two models that have been put forward as explanations of how the *ttg2* mutation can lead to early endosperm cellularisation; the integument size restriction model and the cellularisation signalling model. Firstly, the 'integument size restriction model', indicates that the decrease in the expansion of the integument cell in *ttg2* limits the size of the embryo sac (Garcia *et al.*, 2005). This reduction of size could increase the concentration of a cellularisation factor such that this development is activated sooner in *ttg2* seeds than

in wild-type seeds (Garcia *et al.*, 2005). However, Dilkes *et al.* (2008) have shown that the dimensions of the embryo sac and the 'rescue' of paternal excess which happens in interploidy crosses are not strongly correlated; thus, an alternative mechanism seems likely. Secondly, in the 'cellularisation signalling model', a cellularisation 'signal' moves between the integuments and the endosperm. In the flavonoid biosynthetic *ttg2* mutant, the transport of this cellularisation signal happens more easily, triggering early endosperm cellularisation (Scott *et al.*, 2013). It is a matter of conjecture as to whether the mechanism of the transport is simply related to biophysical changes in the endothelium that change its permeability to biomolecules or whether active processes may be involved. Dilkes *et al.* (2008) give preference to the cellularisation signalling model. Further evidence points to a role for the FBP, in interploidy crosses, a mutation in the *TRANSPARENT TESTA 4* (*TT4*) gene from a L*er* ecotype as mother parent with a Col4*x* pollen parent reported high frequencies of the rescue seeds (Scott *et al.*, 2013). *TT4* encodes the enzyme chalcone synthase which is the first committed step of the pathway (Figure 1.9). The rescue seed was marked by a highly and early cellularised endosperm as seen in *ttg2* X Col4x crosses (Doughty *et al.*, 2014).

1.6.2. Genetics and characterisation of the flavonoid biosynthesis pathway

1.6.2.1 Introduction

Flavonoids, a diverse family of plant secondary metabolites (Over 6000 different types), are obtained from phenylalanine by the general phenylpropanoid and malonyl-CoA, generated from citrate (Harborne and Williams, 2000, Romeo and Dixon, 2002; Kleindt *et al.*, 2010). Flavonoids play an important role in the colour and flavour of fruits, vegetables, seeds and flowers; also in attraction of pollinators, regulation of polar auxin transport, protection against ultraviolet radiation, defence against pathogens and herbivores, and have a physiological role in seed dormancy or viability (Moise *et al.*, 2005; Kleindt *et al.*, 2010; Thompson *et al.*, 2010). In addition, flavonoids are valuable to human health because they are known to have beneficial antioxidant properties (Moise *et al.*, 2005).

There are three major flavonoid subgroups in *A. thaliana*: flavonols, anthocyanins, and proanthocyanidins (condensed tannins/PAs) (Kleindt *et al.*, 2010; Appelhagen *et al.*, 2014) (Figure 1.9). For example, in CHS, CHI, F3H, and DFR co-localise on the endoplasmic reticulum (ER) (Lepiniec *et al.*, 2006). Moreover, it is believed that most

structural enzymes of the FBP work specifically on the endoplasmic reticulum membrane within the cytosol (Burbulis and Winkel-Shirley, 1999).

1.6.2.2 The biosynthesis of flavonoids

Flavonoids are obtained from the main substrates (i) phenylalanine, which is produced through the shikimate pathway and (ii) malonyl-CoA generated by the carboxylation of acetyl-CoA and catalysed by the acetyl-CoA carboxylase (Romeo and Dixon, 2002; Kleindt *et al.*, 2010). Flavonoid biosynthesis products are transported to different extracellular or sub-cellular areas, and then carried into the vacuole (Andersen and Markham, 2006) (Figure 1.9).

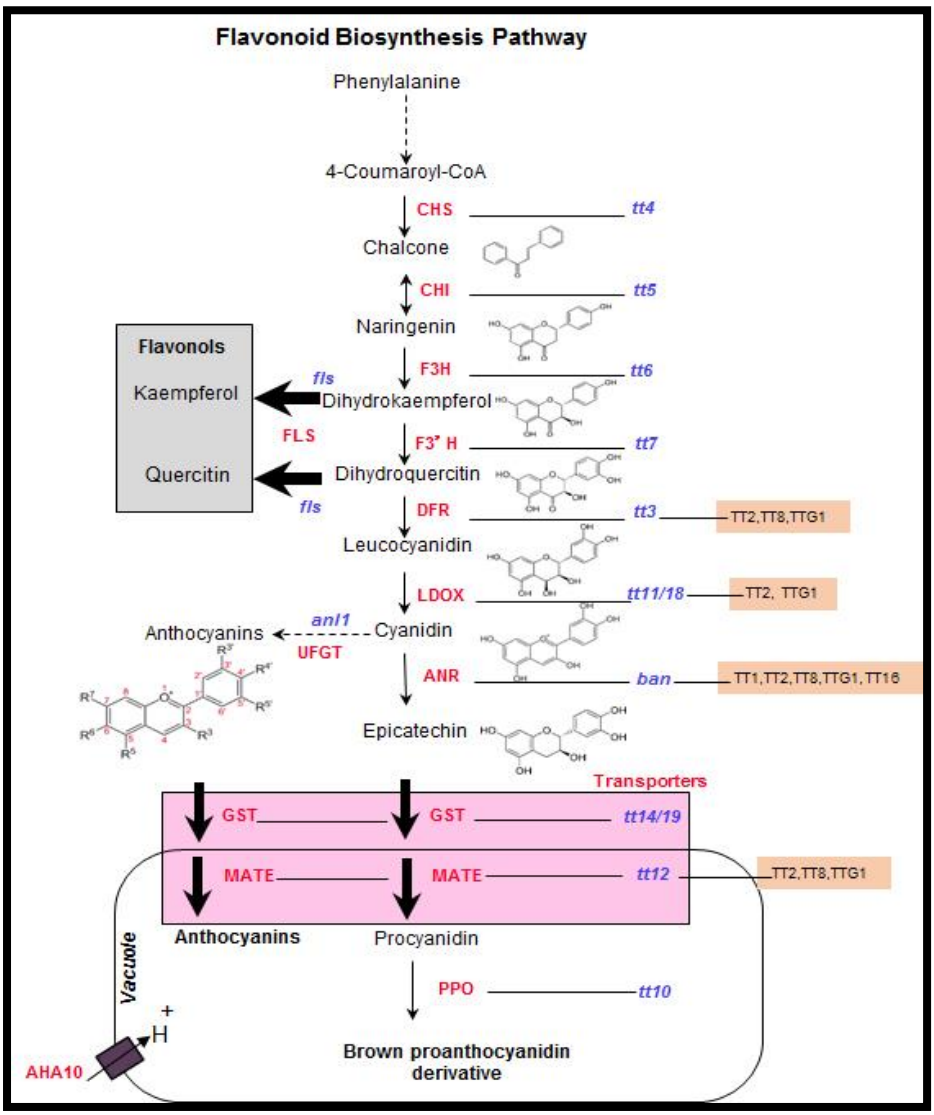


Figure 1.9: The flavonoid biosynthesis pathway in *A. thaliana* seeds. The FBP is a series of reactions converting phenylalanine into flavonols, anthocyanin and proanthocyanidin, which accumulate in the vacuole. The *tt* mutants are shown in blue. The pink box shows transporters into the vacuole. The vacuole is indicated by the black box. Moreover, multiple steps illustrate by dashed arrows. Orange boxes display transcription factors. The white box indicates the vacuole. Enzymes are shown with a red bold font and their names are: **CHS:** Chalcone synthase; **CHI:** Chalcone isomerase**; F3H:** Flavanone-3-hydroxylase**; F3'H:** Flavonoid-3'-hydroxylase**; FLS:** Flavonol synthase**; DFR:** Dihydroflavonol 4- reductase**; LDOX:** Leucoanthocyanidin dioxygenase**; UGFT:** UDP-glucose: flavonoid 3-O-glucosyltransferase**; ANR:** Anthocyanidin reductase**; GST:** Glutathione S-transferase**; MATE:** MATE secondary transporter; **AHA10:** Autoinhibited H^+-ATPase 10; **PPO:** Polyphenol oxidase (modified from Winkel-Shirley, 2001; Debeaujon *et al.*, 2003; Baxter *et al.*, 2005; Pourcel *et al.*, 2005; Lepiniec *et al.*, 2006; Kleindt *et al.*, 2010; Gou *et al.*, 2011; Appelhagen *et al.*, 2014).

1.6.2.3 Anthocyanins

Anthocyanins often occur in high concentrations, are visible to the human eye and are water-soluble plant pigments, and, were the first flavonoids to be examined (Andersen and Markham, 2006). Most of the purple anthocyanins that are found in the seed coat, siliques, stems and leaves have proved to be cyanidin derivatives (Abrahams *et al.*, 2003; Routaboul *et al.*, 2006). Anthocyanins are clear in 4-day-old seedlings and also in older plants that exhibit chlorophyll degradation (Bradford *et al.*, 2007). Anthocyanin structures are composed of the aglycone backbone (anthocyanidin), sugar(s), and acyl group(s) -in some cases- (Chopra *et al.*, 2006).

Anthocyanins perform an important function creating the flower coloration that attracts pollinators. Many factors have been confirmed as affecting the colour of anthocyanins such as form and concentration of the anthocyanidin, salts and pH (Brouillard and Dangles, 1994; Delgado-Vargas *et al.*, 2000). Anthocyanins are typically synthesised in the cytoplasm, and then transported across the tonoplast and into the vacuolar space (Zhang *et al.*, 2006) as has been demonstrated in maize (Marrs *et al.*, 1996) and grapevine (Gomez *et al.*, 2009). Furthermore, anthocyanins have been discovered within anthocyanoplasts (APIs), that are cytoplasmic vesicles and may be accountable for conveying anthocyanic vacuolar inclusions (AVIs) into that cell compartment (Braidot *et al.*, 2008). It has been suggested that the pivotal importance of anthocyanins on the intensity of flower colour is independent of any presence of AVIs in the cells (Markham *et al.*, 2000).

In many plants, the accumulation of anthocyanin may be stimulated by sugars (sucrose-dependent signalling pathway) (Das *et al.*, 2012).

1.6.2.4 Flavonols

Flavonols exist mainly as glycoside derivatives that are especially located in the palisade layer (Oi1) of the seed coat, the endosperm, and the embryo (Figure 1.10.A and B) (Pourcel *et al.*, 2005; Routaboul *et al.*, 2006; Bradford *et al.*, 2007). Quercetin-3-*O*-rhamnoside (Q-3-O-R) is the main flavonol in mature seeds although a comparatively newly identified group of biflavonols, quercetin-rhamnoside dimers, has also been detected. Q-3-O-R and biflavonols are both located mainly in the testa (Bradford *et al.*, 2007; Routaboul *et al.*, 2006). In most plants, derivatives of the three most significant flavonols i.e. quercetin, myricetin and kaempferol are synthesised. Each has an important

part to play in various biological processes, yet they differ by only an individual hydroxyl group on the B ring (Figure 1.11) (Owens *et al.*, 2008). Flavonols protect plants against UV light, and can regulate auxin transport within the seedling (Emiliani *et al.*, 2013).

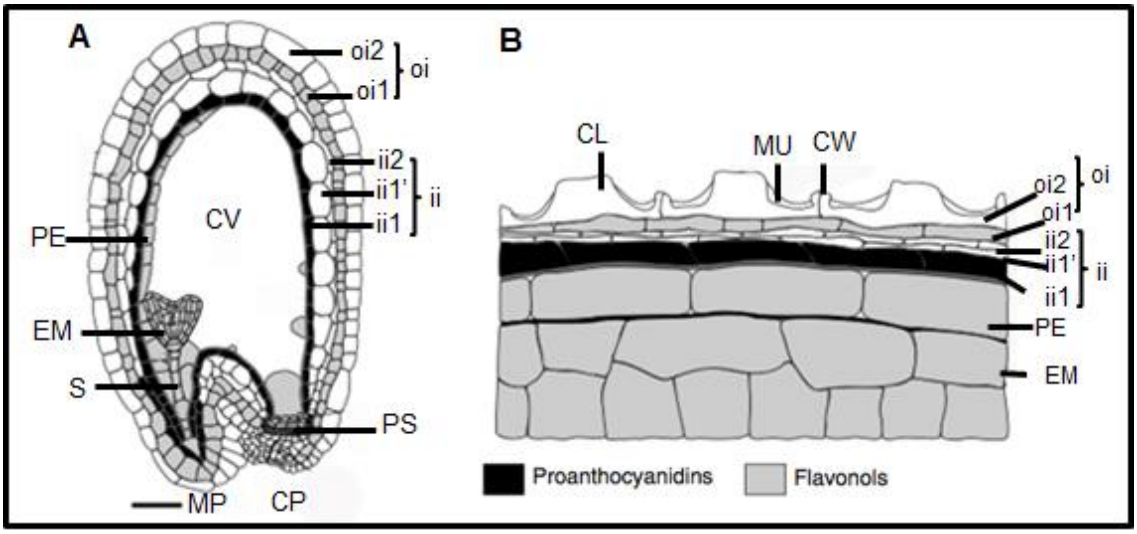


Figure 1.10: Localisation of flavonoids and seed coat structure in *A. thaliana* seeds. A. Longitudinal section of the developing seed anatomy with the embryo at heart stage. Cells accumulating proanthocyanidins are shown as black and those accumulating flavonols are shown as grey. **B.** The mature testa is shown in cross section. **Abbr.: CL**: columella; **CP:** chalazal pole; **CV**: central vacuole; **CW**: cell wall; **EM**: embryo; **ii**: inner integument; outer layer of inner integument layer (**ii2**); median layer of inner integument layer (**ii1'**) and pigment layer is also called 'endothelium' (**ii1**); **MP**: micropylar pole; **MU**: mucilage; **oi**: outer integument; **oi2**: the outer epidermis of outer integument layer; **oi1:** inner epidermis of outer integument layer 'palisade'; **PE:** peripheral endosperm; **PS**, pigment strand; **S**, suspensor. The scale bars are 40 μm in (**A**), and 7 μm in (**B**) (modified from Bradford *et al.*, 2007).

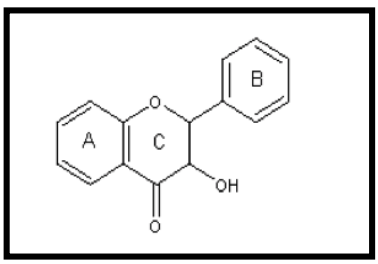


Figure 1.11: Shows the structure of general flavonol (Kumar and Pandey, 2013).

1.6.2.5 Proanthocyanidins

Before forming the PAs, flavonoid polymers of the flavan-3-ol epicatechin (EC, 2-3-*cis*) would be found in the testa of *A. thaliana* seeds. These colourless flavonoid polymers are called procyanidins and are the result of condensation of flavan-3-ols (Pourcel *et al.*, 2005; Routaboul *et al.*, 2006; Appelhagen *et al.*, 2014). This step is followed by oxidation

of Flavan-3-ols leading to the formation of oxidised brown proanthocyanidins (tannin) (David, 2014). The synthesis of colourless condensed tannins occurs in vesicles that are created from the endoplasmic reticulum and which further integrate into the central vacuole (Stafford, 1988). PAs are found largely in the chalaza (pigment strand) and the endothelium layer of the inner integuments (Figure 1.10), where it acts as a part of the plant's defence against disease and playing a role in seed dormancy (Debeaujon *et al.*, 2003).

It is noteworthy to mention at this stage that reverse genetic approaches have been used –originally- to describe the biosynthetic pathway leading to PA accumulation in *A. thaliana* using *tannin-deficient seed* (*tds),* the *transparent testa (tt)* and *banylus* (*ban*) (Albert *et al.*, 1997; Abrahams *et al.*, 2003).

1.6.2.6 Transparent testa mutants

In *A. thaliana,* PA mutants can be easily identified because single copy genes encode all known enzymes of the central flavonoid pathway (Appelhagen *et al.*, 2014). However, this is not the case for the genes encoding flavonol synthases (FLSs) (Owens *et al.* 2008; Appelhagen *et al.*, 2014), and – possibly -anthocyaninless1 (ANL1) (Kubo *et al.*, 2007). FLSs or glycosyltransferases are necessary for the forming of flavonol glycosides and anthocyanins, respectively (Appelhagen *et al.*, 2014).
In order to validate these previous studies on potential gene redundancy for the FBP a BLAST analysis was carried out for all the FBP genes utilised in this thesis (Table 1.4). This confirmed these studies with only *FLS* and *ANL1* genes having homologues that could contribute to FBP function.

Table 1. 4: Flavonoid Biosynthesis Pathway genes largely encoded by single copy genes except *fls* and *anl1* mutants.

Gene	Gene family	Putative homologous gene sequence (% identity | coverage to query sequence)	Evidence of gene expression in seed?	Reported function of Homologous gene(s)	Published evidence for redundancy
TT4(At5G13930), Chalcone synthase	Chalcone and stilbene synthase family protein	• At1g02050 (38 % | 0.98) • At4g00040 (38 % | 0.95) • At4g34850 (38 % | 0.94)	• No. • Yes very low expression during seed development. • No	• Hydroxyalkyl alpha-pyrone synthase (AtPKSb/AtLAP6) • Putative chalcone synthase • Hydroxyalkyl alpha-pyrone synthase (AtPKSa/AtLAP5)	No (Appelhagen *et al.*, 2014).
TT5(At3G55120), Chalcone isomerase	Chalcone-flavanone isomerase family protein	• At5g66220 (52 % | 0.70) • At5g05270 (25 % | 0.83) • At1g53520 (20 % | 0.69)	• Yes medium level of expression during seed development. • Yes high level of expression. • Yes high level of expression at torpedo and linear cotyledon stages.	• Putative chalcone-flavonone isomerase • Chalcone isomerase (AtCHI) • Putative chalcone-flavonone isomerase	No (Appelhagen *et al.*, 2014).
TT6(At3G51240), Flavanone-3-hydroxylase	Flavanone 3-hydroxylase	• At2g38240 (33 % | 0.87) • At4g10490 (33 % | 0.86) • At4g10500 (32 % | 0.92)	• No. • Yes high level of expression at heart, torpedo and linear cotyledon stages. • No	• Putative 2OG-Fe(II) oxygenase • Putative DMR6-like oxygenase (AtDLO2) • Putative salicylic acid 3-hydroxylase(AtDLO1/AtS3H)	No (Appelhagen *et al.*, 2014).

TT7(At5G07990), Flavonoid-3' -hydroxylase	Cytochrome P450 monooxygenase involved in flavonoid biosynthesis	No	No	No	No (Appelhagen *et al.*, 2014).
TT3 (At5G42800), Dihydroflavonol 4-reductase	Dihydroflavonol-4-reductase	• At4g35420 (40 % | 0.86) • At2g45400 (39 % | 0.85) • At2g33600 (38 % | 0.84)	• No. • Yes high level of expression at torpedo stage. • Yes medium level of expression during seed development.	• Tetraketide alpha-pyrone reductase (AtTKPR1) • Putative dehydrogenase, involved in brassinosteroid (AtBEN1) • Putative NAD-dependent epimerase/dehydratase	No (Appelhagen *et al.*, 2014).
FLS (At5G08640), Flavonol synthase	Flavonol synthase	• At5g63590 (65 % | 0.88) • At5g43935 (56 % | 0.88) • At5g63600 (53 % | 0.98) • At5g63580 (49 % | 0.66) • At5g63595 (48 % | 0.84)	• Yes high level of expression at linear cotyledon stage. • No • No • Yes medium level of expression at pre-globular, globular and heart stages. • No	• Putative flavonol synthase (AtFLS3) • Putative flavonol synthase (AtFLS6) • Putative flavonol synthase (AtFLS5) • Putative flavonol synthase (AtFLS2) • Putative flavonol synthase (AtFLS4)	Yes (Owens *et al.*2008; Appelhagen *et al.*, 2014).

TT11/TT18/TDS4-4 (At4G22880), Leucoanthocyanidin dioxygenase	Leucoanthocyanidin dioxygenase family protein	• At4g22870 (40 % | 0.30) • At5g08640 (40 % | 0.95) • At5g63590 (36 % | 0.83)	• Yes high level of expression at pre-globular and globular stages and at heart and torpedo stages medium level of expression. • Yes very low expression during seed development. • Yes high level of expression at linear cotyledon stage.	• Anthocyanidin synthase (AtANS) • Flavonol synthase (AtFLS1) • Putative flavonol synthase (AtFLS3)	No (Appelhagen *et al.*, 2014).
BAN (At1G61720), Anthocyanidin reductase	Anthocyanidin reductase	• At4g35420 (39 % | 0.96) • At1g76470 (37 % | 0.97) • At2g33590 (37 % | 0.96)	• No. • Yes very low expression during seed development. • No.	• Tetraketide alpha-pyrone reductase (AtTKPR1) • Putative NAD-dependent epimerase/dehydratase	No (Appelhagen *et al.*, 2014).
ANL1 (At5G17050), UDP-glucose: flavonoid 3-O-	UDP-GLUCOSYL TRANSFERASE 78D2 (UGT78D2)	• At5G17030 (76 % | 1.00) • At1G30530 (72 % | 1.00) • At5G17040 (71 % | 0.98)	• No. • Yes high level of expression during seed development. • Yes high level of expression at torpedo and linear cotyledon stages.	• UDP-dependent glycosyl transferase (AtUGT78D3) • UDP-dependent glycosyl transferase (AtUGT78D1) • Putative UDP-dependent glycosyl transferase	Yes **(**Kubo *et al.*, 2007; not considered by Appelhagen *et al.*, 2014).

TT14/TT19 (At5G17220), Glutathione S-transferase	Glutathione S-transferase phi 12	• At3g03190 (69 % | 1.00) • At2g30860 (41 % | 0.96) • At4g02520 (40 % | 0.96)	• No. • Yes very low expression at globular and heart stages. • No.	• Putative class phi glutathione S-transferase (AtGSTF11) • Putative class phi glutathione S-transferase (AtGSTF9) • Putative class phi glutathione S-transferase (AtGSTF2)	No (Appelhagen *et al.*, 2014).
TT12 (At3G59030), MATE secondary transporter	Multidrug and Toxin Extrusion (MATE) family	• At3g21690 (48 % | 0.98) • At1g47530 (44 % | 0.92) • At1g61890 (43 % | 0.92)	• Yes very low expression at globular and heart stages. • Yes medium level of expression during seed development. • Yes medium level of expression during seed development.	• Putative MATE-related efflux carrier (AtDTX40) • Putative MATE-related efflux carrier (AtDTX33) • Putative MATE-related efflux carrier (AtDTX37)	No (Appelhagen *et al.*, 2014).
TT10 (At5G48100) (AtLAC15), Polyphenol oxidase	Laccase/Diphenol oxidase family protein	• At5g09360 (49 % | 0.97) • At5g05390 (47 % | 0.99) • At1g18140 (46 % | 0.98)	• Yes very low expression at pre-globular, heart, torpedo and linear cotyledon stages. • Yes very low expression at torpedo and linear cotyledon stages. • No.	• Putative laccase (AtLAC14) • Putative laccase (AtLAC12) • Putative laccase (AtLAC1)	No (Appelhagen *et al.*, 2014).

AHA10 (At1g17260), Autoinhibited H^+-ATPase 10	Autoinhibited H(+)-ATPase isoform 10	• At3g42640 (71 % | 0.99) • At3g47950 (71 % | 0.98) • At5g62670 (71 % | 0.98)	• No. • Yes medium level of expression at pre-globular, heart stages but at torpedo and linear cotyledon stages the level of expression is increased. • Yes medium level of expression during seed development.	• Putative plasma membrane proton-transporting P3A-type ATPase (AtAHA8) • Putative proton-transporting P3A-type ATPase (AtAHA4) • Putative plasma membrane proton-transporting P3A-type(AtAHA11)	No (Appelhagen *et al.*, 2014).
TTG2 (At2G37260)	WRKY family transcription factor family protein	• At2g38470 (30 % | 0.72) • At4g26640 (29 % | 0.74) • At5g07100 (26 % | 0.62)	• No. • Yes high level of expression during seed development. • No.	• Putative WRKY-type transcription factor (AtWRKY33) • Putative WRKY-type transcription factor (AtWRKY20) • Putative WRKY-type transcription factor (AtWRKY26)	No (Appelhagen *et al.*, 2014).

The evidence of gene expression during seed development is available in Arabidopsis eFP Browser. Three top homologues blast are high indicated with reported function according to ARAMEMNON database.

1.6.2.6.1 Genes important in flavonoid biosynthesis

Several mutants of FBP genes have been characterised (Appelhagen *et al.*, 2014; Satio *et al.*, 2013). FBP mutants fall into three major groups; *transparent testa* (*tt*), *transparent testa glabra* (*ttg1, ttg2*) and *tannin-deficient seeds (tds)* (Shirley, 1998; Pourcel *et al.*, 2005; Lepiniec *et al.*, 2006). These mutations affect the levels of various pigmented flavonoid compounds, leading to changes in the seed coat colour of *tt*, *ttg* and *tds* mutants. These colour changes can range from yellow (*tt1– tt5, tt8, ttg1, tds4/tt18/tt11, tt16* and *ttg2*) to pale brown (*tt6, tt7, tt19/14, tt10, tt12* and *aha10*) (Appendix A). *ban* mutant has unusually coloured spotted, gray-green mature seeds, due to the accumulation of anthocyanin pigments in the endothelium of immature seeds (Albert *et al.*, 1997; Devic *et al.*, 1999; Debeaujon, 2000; Lepiniec *et al.*, 2006; Appelhagen *et al.*, 2014).

Genes that encode biosynthetic enzymes of the FBP include *TT4, TT5, TT6, TT7, TT3, FLS, TDS4/TT18/TT11, BAN* whereas *TT19/TT14, TT12* and *AUTOINHIBITED H^+-ATPase ISOFORM 10 (AHA10*) encode proteins that are responsible for determining the localisation of FBP products within the cell. As introduced above there is no functional redundancy for these genes with the exception of *FLS* where multiple genes encode the enzyme responsible for catalysing this branch point leading to flavonols (Lepiniec *et al.*, 2006). As flavonols play no significant part in the pigmentation of the seed coat, and/or as the genes are functionally redundant, no mutant *tt* phenotype for *FLS* has been detected (Romeo and Dixon, 2002).

ANTHOCYANINLESS1 (ANL1) is a homeobox gene (a large family of similar genes that during early embryonic development direct the formation of several body structures) that affects anthocyanin distribution in *A. thaliana*. It was discovered by studying anthocyaninless mutants along with the genes that produce anthocyanin (Burdzinski and Wendell, 2007). Kubo *et al.* (2007) noted that *anl1* has normal seed colour but a reduced accumulation of anthocyanin.

1.6.2.6.2 The transport of secondary metabolites by membrane transporters

TT12 (a putative multidrug and toxic compound extrusion transporter), TT19 (glutathione-S-transferase) and *AHA10* (encodes an H^+-ATPase) have been shown to play a role in targeting of the PA precursors (flavan-3-ols) to the vacuole (Haughn and

Chaudhury, 2005). For example, *TT12* was recently shown to encode an epicatechin 3'-O-glucoside/H+ antiporter sited in the vacuolar membrane (Figure 1.9). This indicates that epicatechin requires glycosylation prior to transport from the cytoplasm to the vacuole, and that this movement is powered by a proton gradient. This proton gradient is likely produced by the action of *AHA10*, a P-type H^+ - ATPase, which acidifies the vacuole thus permitting flavan-3-ol import. It has been noticed that mutations in vacuole acidification are affected seed colour (Appelhagen *et al.*, 2014). In 2005, Moise *et al.* reported a list of flavonoid genes in *A. thaliana* (Table 1.5).

The role of *TT19* is as yet unclear; however, a knockout mutation leads to a reduction in PA levels, suggesting that it may play a role in alternative flavonoid transport into the vacuole, or even long-range flavonoid transport. Interestingly, all *tt12, tt19* and *aha10* plants are defective in vacuolar biogenesis. This is indicated by the accumulation of many small vacuoles as opposed to one large one (Moise *et al.*, 2005). Thus, in summary, mutations in these genes (*TT12, TT19* and *AHA10*) cause a disruption of PA biosynthesis by blocking vacuolar biogenesis in the endothelium that imports PA precursors (Haughn and Chaudhury, 2005; Zhao *et al.*, 2010; Appelhagen *et al.*, 2014).

Table 1.5: FBP genes that are expressed in the seed coat (adapted from Moise *et al.*, 2005).

Flavonoid biosynthesis genes	Locus	Enzyme	Literature
TT4	AT5G13930	Chalcone synthase	Feinbaum and Ausubel, 1988
TT5	AT3G55120	Chalcone isomerase	Shirley *et al.*, 1992
TT6	AT3G51240	Flavonone 3-hydroxylase	Wisman *et al.*, 1998
TT7	AT5G07990	Flavonone 3′-hydroxylase	Schoembohm *et al.*, 2000
TT3	AT5G42800	Dihydroflavonol 4-reductase	Shirley *et al.*, 1992
FLS1	AT5G08640	Flavonol synthase	Owens *et al.*, 2008
TT18/ TT11/ TDS4	AT4G22880	Leucoanthocyanidin dioxygenase	Abrahams *et al.*, 2003
BAN	AT1G61720	Anthocyanidin reductase	Devic *et al.*, 1999
ANL1	AT5G17050	UDP-glucose:flavonoid-3-o-glucosyltransferase	Kubo *et al.*, 2007
TT19/ 14	AT5G17220	Putative glutathione S-transferase	Kitamura *et al.*, 2004
TT12	AT3G59030	Multidrug secondary transporter-like protein	Debeaujon *et al.*, 2001
TT10	AT5G48100	Laccase-Like polyphenol oxidases	Pourcel *et al.*, 2005
AHA10	AT1G17260	Plasma membrane H^+-ATPase	Baxter *et al.*, 2005
TT16	AT5G23260	MADS box protein	Nesi *et al.*, 2002
TTG2	AT2G37260	WRKY domain protein	Johnson *et al.*, 2002

1.6.2.7 Regulation of flavonoid biosynthesis by MYB and other transcription factors

Transcriptional control of flavonoid biosynthesis genes occurs mainly in response to biotic and abiotic stress, utilising a range of transcription factors, like MYB (Weisshaar and Jenkins, 1998). MYB TFs are classified into three main groups: R1R2R3-MYB, R2R3-MYB and the MYB-related proteins - a heterogeneous group usually characterised by one MYB repeat (Stracke *et al.*, 2001). The formation of flavonol is controlled by the R2R3-MYB proteins PFG1 to 3 (Stracke *et al.*, 2007), whereas a network involving the joint action of MYB, basic helix loop helix (bHLH) and WD40 repeat proteins (MBW) regulate the biosynthesis of PA and anthocyanin (Appelhagen *et al.*, 2014). In details, in young *A. thaliana* seedlings, a ternary complex of a) MYB75/PAP1, b) BHLH042/TT8 or BHLH002/EGL3 and c) WD40/TTG1, is pivotal for the accumulation of anthocyanin (Appelhagen *et al.*, 2014).

As for PA, Baudry *et al.* (2004) reported that in seeds, the major regulator of PA metabolism is a complex of MYB123/TT2, TT8 and TTG1. Xu and his co-workers (2014) showed that three other MBW complexes were involved in the expression of PA biosynthesis genes in specialised tissues of the seed coat. TT1, TT2, TT8, TT16, TTG1, and TTG2 are transcription factors that regulate PA biosynthesis. TT2, TT8, TTG1 are the first group of regulators: a) TT2 is an endothelium-specific (MYB transcription factor) that is essential for PA biosynthesis (Figures 1.9 and 1.12) (Debeaujon *et al.*,

2003; Haughn and Chaudhury, 2005), b) TT8 is a bHLH transcription factor and c) TTG1 is a WD40 repeat protein (Debeaujon *et al.*, 2003; Lepiniec *et al.*, 2006).

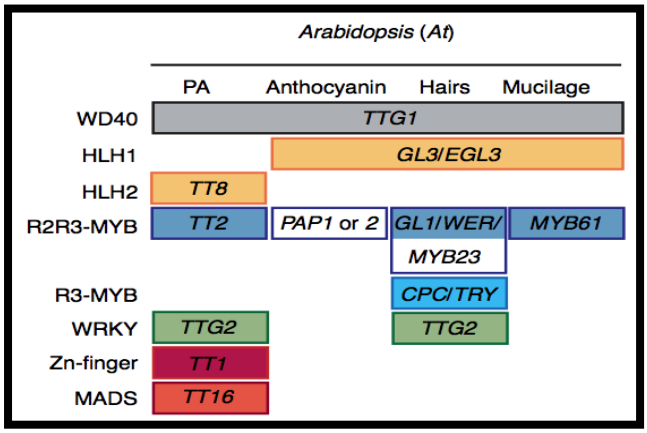


Figure 1.12: Illustration of the genetic control of the pigments in *A. thaliana*. The bars indicate transcription factors, controlling hair development, the production of seed mucilage, Anthocyanin, and PA (adapted from Koes *et al.*, 2005).

TT1 (a WIP zinc-finger protein, which mainly expressed in the endothelium layer during seed development), and TT16 (an ARABIDOPSIS BSISTER MADS box protein, whose expression is restricted to the developing seed and early flower) are a second group of regulators of the FBP. The cell morphology of the endothelium layer in *tt1* and *tt16* plants appears aberrant which suggests that both genes control differentiation of the inner integument cells and are of critical importance for correct development of the endothelial cell type (Nesi *et al.*, 2002; Sagasser *et al.*, 2002; Debeaujon *et al.*, 2003; Moise *et al.*, 2005).

Finally, the third group of regulators contains TTG2/WRKY44, which is a zinc finger transcription factor; TTG2 controls PA biosynthesis -similar to TT2, TT8 and TTG1- in the whole seed coat downstream of TTG1 (Johnson, 2002; Debeaujon *et al.*, 2003; Lepiniec *et al.*, 2006).

1.7. Regulation of endosperm development - a maternal cellularisation signal

Thus, by combining our knowledge from the effect of the triploid block and the molecular players in the FBP, it could be hypothesised that a functional FBP inhibits transport of a cellularising factor between the maternal integuments and the endosperm in *A. thaliana* interploidy cross (2xX4x). This would be referred to as the maternal cellularisation signal hypothesis (Scott, pers. comm.).

So, whilst in normal balanced crosses (2xX2x) the cellularisation factor directs cellularisation at the appropriate developmental time point, 2xX4x crosses extra paternal genomes in the endosperm lead to over-proliferation of this tissue prior to cellularisation leading to bigger seeds (or lethality in the Col-0 ecotype) (Figure 1.13.A). However, *tt* mutants, that disrupt the FBP, allow increased transport of the maternal cellularising factor from the integuments to the endosperm, causing earlier endosperm cellularisation. As a result, this prevents excessive endosperm proliferation that is characteristic of Col4x sperm induced lethality, and therefore, rescues seed lethality (Scott *et al.*, 1998; Tiwari *et al.*, 2010) (Figure 1.13.B).

Furthermore, it is known that the endosperm is a source of phytohormones (Lopes and Larkins, 1993) and it is hypothesised that auxin may be the maternal cellularisation factor, as an independent signal or in relation to flavonoids (Peer *et al.*, 2001; Thompson *et al.*, 2010) (Figure 1.13.B). For example, some intermediates of the FBP (kaempferol and quercetin) have been shown to interfere with polar auxin transport, (Peer *et al.*, 2001; Peer *et al.*, 2004) and auxin flow is increased in *tt4* adult plants (Brown *et al.*, 2001). In addition to this, it is thought that a low level of auxin is required at the start of the development of the endosperm as reported from maize studies. This increases from 9DAP to 11DAP and stays at this high level until the seed matures (Lur and Setter, 1993).

Moreover, some FBP mutants in L*er* ecotype such as *tt3* and *tt7* rescue lethality of Col4x (Scott, pers. comm.) even though they cause accumulation of various flavonoid compounds (*tt3* causes quercetin and kaempferol accumulation, *tt7* causes kaempferol accumulation only), which interact with auxin transport proteins and inhibit auxin transport (Peer and Murphy, 2007). Hence, all this suggests that a) auxin transport is not inhibited enough to prevent earlier cellularisation and rescue, or b) transport may actually occur from the endosperm out to the integuments or c) auxin is not the endosperm cellularisation factor that rescues Col4x lethality. If auxin is not the cellularisation factor then what could be fulfilling this role? Importantly levels of various FBP intermediates are reduced in FBP mutants. For example, PAs may reduce the permeability of integument cells and thus reduce the transport of cellularisation factor (Johnson *et al.*, 2002; Garcia *et al.*, 2005); thus absence of PAs in *ttg2-1* mutants may lead to greater diffusion of a molecule that induces endosperm cellularisation and differentiation (Dilkes *et al.*, 2008), leading to rescue of Col4x lethality.

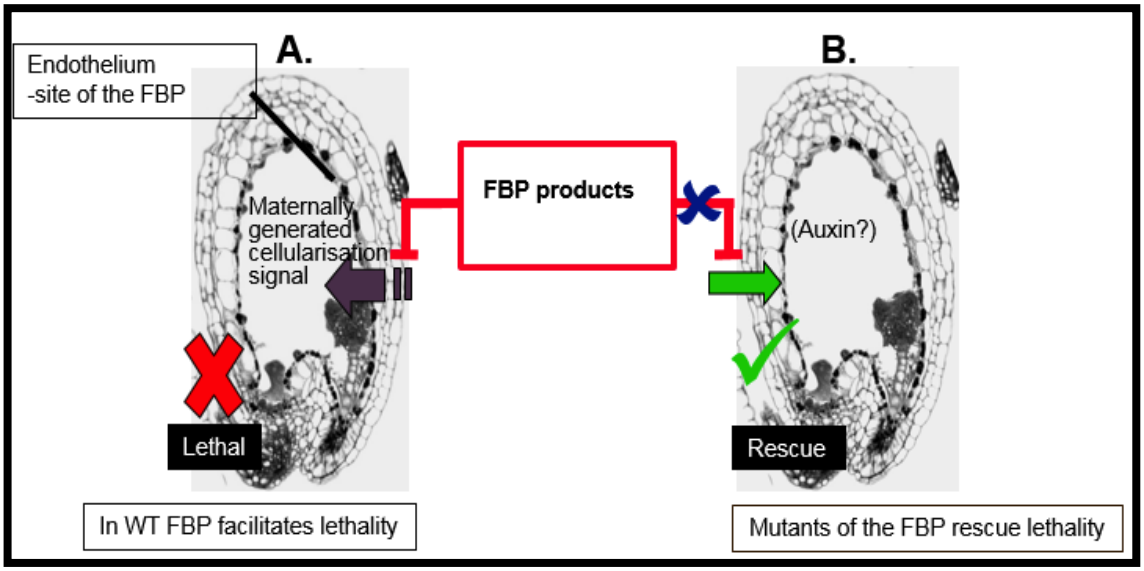


Figure 1.13: Model is showing the effect of FBP products in wild-type and FBP mutant in *A. thaliana*. **A.** Functional FBP inhibits the action of an endosperm cellularisation factor (Col2xXCol4x). The FBP is present in an endothelium layer (the ii1 layer of maternal inner integument layers). The model predicts that the maternal integuments layers produce a maternal cellularisation factor that is transported into the endosperm through the endothelium (purple arrow). The presence of FBP (red box) in the endothelium layer inhibits this signal. Therefore, endosperm cellularisation does not occur or is significantly late. Delayed cellularisation allows lethal over-proliferation of the endosperm as is seen in Col2xXCol4x crosses. **B.** FBP mutants alleviate inhibition/transport of the cellularisation factor. Introducing a *tt* mutation (blue x) causes disruption of the FBP (red box) in the endothelium layer. This prevents or causes a reduction in the production of the final products of this pathway (e.g. PAs). Therefore, the transport of the cellularisation factor through the maternal integuments layers to the endosperm is increased (green arrow). As a result endosperm cellularisation occurs early, rescuing Col4x-mediated seed lethality (Scott, pers. comm.).

1.8. Project aims and objectives

This study aims to investigate the role of the FBP and its intermediates in regulating paternal Col4x-induced seed lethality ('Col-killer') in *A. thaliana*. Below is a summary of the objectives and a brief description of methods used to address these:-

1. **Verify the role of the FBP in the Col-killer effect**

Previous preliminary investigations into the phenomenon used FBP mutants in a mixture of ecotypes (Columbia, Landsberg *erecta* and Wassilewskija), potentially complicating the interpretation of the role of the FBP. This objective was designed to achieve consistency of genetic background by conducting all interploidy crosses (2x FBP mutant seed parent X wild type 4x pollen) in the Col-0 background.

2. **Identify the FBP intermediates responsible for regulating the Col-killer effect**

This objective uses a genetic approach (*transparent testa* double mutants) to alter the flux of FBP intermediates to determine which classes of flavonoids are involved in Col4x induced seed lethality.

3. **Verify the FBP intermediates responsible for regulating the Col-killer effect**

This objective will quantify the effect of a selection of *tt* mutants in the Col-0 and L*er* ecotypes on the flux and accumulation of flavonoids through the FBP in seeds by mass spectrometry. Additionally, mass spectrometry analysis of auxin levels will also be carried out in the same lines.

4. **Determine whether auxin and the FBP interact in the Col-killer effect**

To understand the potential role of auxin in rescue of Col4x induced seed lethality the auxin response 'reporter' construct DR5::GFP used to measure auxin flux in WT and FBP mutant maternal lines.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Plant material

Ethyl methanesulfonate (EMS) mutant lines used are as follows: *tt4-1, tt10-5* and *ttg2-1* in the Landsberg *erecta* (L*er*) background, *fls-1* in the Columbia (Col-0) background, and *tt12-1* (T-DNA Feldmann, 1992) and *tt16 -1* in the Wassilewskija (Ws) background. Seeds of L*er* 2x mutant lines were provided by the Nottingham Arabidopsis Stock Centre (NASC), UK. Ws2x and Col-0 (Col2x) mutant lines were supplied by Isabelle Debeaujon (INRA, France).

Arabidopsis thaliana (*A. thaliana*) T-DNA insertion lines in the Col-0 background that were used are as follows: (*tt4-12*) GABI_304D03.02, (*tt5-3*) SALK_034145.51.00.x, (*tt6*) SALK_068963.53.50.x, (*tt7)* SAIL_573_G12, (*fls1*) SALK_009992.49.80.x, (*tt3*) SALK_099848.56.00.x, (*tt11/tt18*) SALK_073183, (*tt14/tt19*) SALK_105779, (*anl1*) SALK_049338.56.00.x, *(ban)* SALK_122474.20.75.x, (*tt12*) GK_797D03.03, (*tt10*) GK_146E10.02, *(aha10-4)* SALK_019569.47.20.x, (*ttg2*) SALK_148838.51.70.x. All T-DNA insertion lines (SALK, SAIL and GABI-Kat lines) were grown as described in section 2.2.1 and were obtained from the Nottingham Arabidopsis Stock Centre (NASC), UK. The auxin responsive reporter construct DR5rev::GFP in the Col-0 ecotype (ER-targeted), having the sulfonamide resistance marker was obtained from NASC. L*er* (L*er*2x) and Columbia-0 (Col2x and Col4x) lines were kindly provided by Prof. Rod Scott (University of Bath). Ecotypes referred to as L*er*, Col-0 and Ws are diploid lines; Col2x also represents diploid Col-0 in this thesis.

2.1.2 PCR primer design and synthesis

SIGnAL (http://signal.salk.edu/cgi-bin/tdnaexpress) was used to design primers used for validation of T-DNA insertion lines. Primers for reverse transcription polymerase chain reaction (RT-PCR) were considered by using primer3web version 4.0.0 (http://primer3.ut.ee/). In order to confirm target specificity of primers, the *Arabidopsis* genome was searched using BLAST SIGnAL search (http://signal.salk.edu/cgi-

bin/tdnaexpress). Invitrogen U.K. supplied the primers (refer to information in Table 2.3 and 2.5).

2.1.3 Statistical analyses

Statistical tests were performed by using SPSS version 21 (IBM, USA); non-normally distributed data was analysed using the non-parametric Kruskal-Wallis test to test for a statistically significant difference between the samples (P- value ≤ 0.05). Then, the Mann-Whitney Test was used to define the important differences between each mutant and its control (P- value ≤ 0.05).

The R statistics package, version 3.1.0 Mavericks build 6734 (The R Foundation for Statistical Computing), was used to carry out statistical analyses for data obtained using IMARIS version 6.1.0 (Bitplane, USA) from the auxin reporter lines.

2.1.4 Image capture and processing

2.1.4.1 T-DNA lines

Images of dry seeds and siliques were captured using a Nikon Digital Sight DS-U1 colour camera in conjunction with a Nikon SM2 1500 dissecting microscope utilising a photonic PL2000 light source NIS-Elements F2.30 Software was used for image capture and additional processing was carried out using Adobe Photoshop Elements.

2.1.4.2 Auxin reporter lines

Confocal Laser Scanning Microscopy (Nikon C1 confocal microscope system[1]) using an Argon laser (488nm excitation) was used to capture images of seeds at 20x magnification. To prevent over-saturation and to maximise the ratio of signal: noise the following settings were used for all z-stack and averaged images: Red channel gain: 110, green channel gain: 100 for auxin reporter line. The 'small' pinhole setting was used and 5 μm optical sections were taken at a 1024x1024 resolution. Individual images for Z-stacks were saved as .ids files. IMARIS version 6.1.0 (Bitplane, USA) was then used to reconstruct the z-stack files into 3D renditions. Quantification of the green channel signal

[1] Nikon Eclips 90i microscope, EZ-C1 software Gold Version 3.50, Build 724; Nikon, Japan.

and calculation of the corresponding volume of the seed coat was carried out by manually selecting both the seed surface and the endosperm using IMARIS software in 'surpass' mode (Bitplane, 2008). An average of two to three siliques was inspected for each cross under study and ten seeds were imaged from each silique.

2.1.5 Preparation of sulfonamide resistance media

Plant growth medium consisted of 25 ml of ½ strength MS media (Duchefa Biochemie Murashige & Skoog Medium, 1962) (2.17 g MS/L (Sigma)) at pH 5.8-5.9 solidified by adding 8.2 g/l Phytoagar (Duchefa Biochemie), and supplemented with sulfonamide antibiotic (5 µg/l) (sulfadiazine) (product number S8626 from Sigma– Aldrich).

2.1.6 General liquid chromatography- mass spectrometry and metabolite profiling

Seeds were extracted from five siliques at 5 DAP and placed in 20 µl of fresh extraction buffer consisting of 10% (v/v) methanol, 1% (v/v) acetic acid and 89 ml Mili-Q water containing an umbelliferone internal standard (product number H24003, Sigma, UK) at a final concentration of 3.6 µg/ml. The extraction step involved incubation on ice followed by 15 minutes sonication in an ice water bath. The supernatants from technical replicates were then pooled after centrifugation (10 min at 16,100 x g, 4 °C) followed by filtration through a 0.4µm (RC) syringe filter (Phenomenex, UK); samples were generated in triplicates.

Quantitative analysis of anthocyanin was performed using an Agilent 6420B triple quadruple (QQQ) mass spectrometer (MS/MS) (Technologies, Palo Alto, USA) hyphenated to a 1200 Series Rapid Resolution high-pressure liquid chromatography (HPLC) electrospray ionization triple quadruple (LC-ESI-QQQ-MS/MS) and analysis was as described by Page *et al.,* (2012).

5 µl of sample extract was loaded onto a Zorbax Eclipse Plus C_{18} 3.5 µm, 2.1 x 150 mm reverse-phase analytical column (Agilent Technologies, Palo Alto, USA). The following gradients were utilized: 0 min – 0% B; 1 min – 0% B; 5 min – 20% B; 20 min – 100% B; 25 min – 100% B; 27 min – 0% B; 7 min post time. QQQ source conditions were as follows: gas temperature 350°C, drying gas flow rate 9 l min^{-1}, nebuliser pressure 35 psig, capillary voltage ±4 kV. All ions were scanned in positive ion mode and given a dwell

time of 30 mseconds. A code for abbreviated anthocyanin and flavonoids were shown in Table 2.1 with the fragmentor voltage and collision energies optimised for each compound listed in Table 2.2 (reproduced from Page *et al.*, 2012).

Relative quantification of anthocyanins and flavonoids used previously determined Multiple Reaction Monitoring (MRMs). The codes for anthocyanins A1, F2, 3 and 5 are listed in Table 2.1 below (reproduced from Page *et al.*, 2012).

Table 2.1: Flavonoids identified in *A. thaliana* (Col-0 ecotype) seed developmental tissue at 5 DAP by liquid chromatography–electrospray ionization triple quadruple mass spectrometry (LC-ESI-QQQ-MS/MS) analysis.

Peak	Retention time (min)	Precursor Ion (m/z)		Product Ion (m/z)		Compound name
A1	11.19	889	[M]+	287	[Cy]+	Cyanidin 3-O-[2″-O-(xylosyl) 6″-O-(p-coumaroyl) glucoside] 5-O-glucoside
				449	[Cy+Glc]+	
				727	[Cy+Glc+Xyl+Cou]+	
F2	11.28	565	[M]+	287	[Km + H]+	Kaempferol [(pentoside)-rhamnoside]
				433	[Km + Rha + H]+	
F3	11.47	579	[M]+	287	[Km + H]+	Kaempferol 3-O-rhamnoside 7-O-rhamnoside
				433	[Km + Rha + H]+	
F5	10.48	741	[M]+	287	[Km + H]+	Kaempferol 3-O-[6″-O-(rhamnosyl) glucoside] 7-O-rhamnoside
				433	[Km + Rha + H]+	
				595	[Km + Rha + Glc + H]+	

Table 2.2: Multiple Reaction Monitoring (MRM) settings for each compound of interest.

Compound Name	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Fragmentor (V)	Collision Energy (V)
A1	889	449	100	35
Flavonoid 2	565	287	100	25
Flavonoid 3	579	287	100	15
Flavonoid 3	579	433	100	15
Flavonoid 5	741	287	100	25
Flavonoid 5	741	433	100	25
Isorhamnetin-di-rhamnoside	609	317	100	35
Isorhamnetin-hexoside-rhamnoside	625	317	100	35
Isorhamnetin-rhamnoside	463	317	100	35
Kaempferol	287	287	100	0
Kaempferol-3, 7-di-O -rhamnoside	579	287	100	35
Kaempferol-3, 7-di-O -rhamnoside	579	433	100	35
Kaempferol-3-O -glucoside-7-O -rhamnoside	595	287	100	35

Kaempferol-3-O -glucoside-7-O -rhamnoside	595	433	100	35
Kaempferol-rhamnoside	433	287	100	35
Kaempferol-rhamnoside-hexoside	595	287	100	35
Kaempferol-rhamnoside-hexoside	595	449	100	35
Procyanidin dimer	579	289	100	35
Procyanidin hexamer	1731	289	100	35
Procyanidin hexamer	1731	579	100	35
Procyanidin hexamer	1731	867	100	35
Procyanidin pentamer	1443	289	100	35
Procyanidin pentamer	1443	579	100	35
Procyanidin pentamer	1443	867	100	35
Procyanidin tetramer	1155	289	100	35
Procyanidin tetramer	1155	579	100	35
Procyanidin tetramer	1155	867	100	35
Procyanidin trimer	867	289	100	35
Procyanidin trimer	867	449	100	35
Quercetin-3-O -glucoside	465.1	303	100	35
Quercetin-3-O -rhamnoside	449	303	100	35
Quercetin-di-rhamnoside	595	303	100	35
Quercetin-di-rhamnoside	595	449	100	35
Quercetin-rhamnoside-hexoside	611	303	100	35
Quercetin-rhamnoside-hexoside	611	465	100	35
Umbelliferone	163.1	107	100	25

2.2 Methods

2.2.1 Seed germination and plant growth

Seeds were immersed in 0.1% electrophoresis grade agarose (Invitrogen, UK) and incubated in a fridge at 4°C for 2–3 days. Seeds were germinated in trays that contained Levingtons F2 + S (compost with sand- Scotts, UK). Trays (35cm X 25cm) were watered from the base with tap water followed by a surface treatment with 0.2 g/litre of insecticide (Intercept 70 WG – Scotts, UK). Trays were then covered with a plastic cover, and placed in a Gallenkamp controlled environment room having an 16 /8 hour light/dark cycle. Temperatures were 22°C in the day and 18°C at night, with 70% humidity. Plastic covers were removed after 1 week.

2.2.2 Classification of mature seeds phenotype

Using a light microscope, the mature seeds were separated and characterised as being either plump and burst or shrivelled. Differentiating size and morphology of mature seeds, led to classifying seeds into two classes based on their viability: a) 'plump and burst' seed phenotypes fall into the rescue category, b) 'shrivelled' seed phenotype falls into the lethal non-viable category.

2.2.3 Crosses and controlled pollinations

Emasculation and pollinations were performed with the aid of a dissecting microscope (Leica MZ6). Flower buds were emasculated by hand, and the remaining buds and siliques were removed to avoid any confusion with the self-fertilised siliques. Typically three floral buds on each axis were emasculated. Two days after emasculation, anthers of the pollen parent were gently brushed against the mature stigma taking care not to damage it. Between 3 and 5 siliques were used for each pollination and seeds were collected at 5, 7 and 9 days after pollination (DAP) for microscopical analysis. To allow a study of seed development at different stages using confocal fluorescence microscopy images.

2.2.4 Mature seed collection and seed weight

Mature siliques were collected and stored in 1.5 ml microfuge tubes just before the pod shattering stage. The lids of the microfuge tubes were pierced to allow any residual moisture to escape and were then stored in a polystyrene container (Sigma) at room temperature. Each microfuge tube contained one silique of mature seeds. When siliques were thoroughly dry (around four weeks after pollination), the seeds were removed from siliques that had been stored in microfuge tubes and then counted with the aid of a Nikon SM2 1500 dissecting microscope having a photonic PL2000 light source. Images were taken using a Nikon Digital Sight DS-U1 colour camera using NIS-Elements F2.30 Software. All seeds from each individual silique were weighed using a Mettler PC4400 microbalance (Mettler-Toledo, Leicester, UK). Mean individual seed weights were calculated by dividing total weight by seed number.

$$\text{Average individual seed weight} = \frac{Total\ weight - average\ weight\ of\ weighing\ boat}{seed\ number}$$

In order to have a useful qualitative measure of how effective each FBP mutant is in overcoming Col4x-induced seed lethality a 'rescue ratio' can be calculated using mean mature seed weights and the percentage of plump and burst seeds:-

$$\text{Rescue ratio} = \frac{\%\ plump\ (or\ mean\ weight)\ of\ mutant}{\%\ plump\ (or\ mean\ weight)\ of\ background}$$

2.2.5 Seed germination

Two filter paper discs (Grade1, 85mm, Whatman) were placed in a plastic Petri dish (90 mm) and 3ml of sterile Milli-Q water was added. 40-50 seeds were then sown in the dish and Parafilm was used to seal the plates to decrease water evaporation. At the same time, several Col2x (WT) seeds (known to be viable) were also added to each dish as a control to ensure germination conditions were favourable. Petri dishes were then stored for two days at 4°C before being moved to standard Arabidopsis growth conditions (16 hr day length, 22°C during the day and 18°C during the night in a growth chamber held at 70% humidity). Incubation was for two weeks after which viability was assessed.

2.2.6 Production of double mutant T-DNA insertion lines

In order to generate *anl1/ban* homozygous double mutants firstly *anl1* and *ban* homozygotes were crossed to produce a heterozygous F1 generation for both T-DNA insertions. Then, the double homozygous mutant F2 plants were selected by genotyping them by PCR as described in section 2.2.17. The same process was used to produce *anl1/tt12* and *ban/tt12* homozygous double mutants. Attempts to generate an *fls/anl1* double mutant failed; this could be a due failure to fertilise ovules or more likely that any seeds produced failed to develop properly (non-viable).

2.2.7 Feulgen staining and confocal laser scanning microscopy

Samples were prepared in 1.5 ml microfuge tubes using a protocol adapted from Braselton *et al.* (1996) and described below:

Fixation:

Using a razor blade, *A. thaliana* siliques (3, 5, 7 and 9 DAP) were cut at both ends and kept in 1.5 ml microfuge tubes with a freshly prepared fixative (3:1, absolute ethanol: acetic acid) and stored overnight at 4 °C. Siliques were moved to 70% ethanol and stored there at 4°C for a maximum of 3-4 months (Braselton *et al.,* 1996).

Staining:

Siliques were washed in sterile Milli-Q H_2O three times for 15 min and hydrolysed in 5N HCl for 60 min. The siliques were then washed in sterile Milli-Q H_2O three times for 10 min. Subsequently, using Schiff's fuchsin-sulfite reagent (4.6 g/L pararosaniline and 7.3 g/L of sodium metabisulfite in 0.1 M HCl, product number S5133 from Sigma– Aldrich) siliques were washed and immersed in Schiff's for 2-3 hours in the dark for staining. The siliques were then washed with cold tap water three times for 10 min (Braselton *et al.*, 1996).

Embedding:

Following staining, the siliques were incubated for 10 min each in increasing concentrations of ethanol - once in 70% (v/v) and 95 % (v/v) ethanol and twice in 100 % (v/v) ethanol and then stored at 4 °C overnight. The following day the 100% ethanol was changed every hour until it was clear (the pink color had disappeared). Pods were then either stored in ethanol at -20°C for several weeks or transferred to 1:1 ethanol: LR White acrylic resin (product number L9774, Sigma–Aldrich) for 1 hour. Following the final wash, 1:1 ethanol: LR White Resin was replaced with pure resin and incubated for 1 hour, and again fresh pure resin was added before storing overnight at 4 °C. A small drop of resin LR White was placed in the middle of a slide, and two coverslips were pushed partway into it from either side. Under a dissecting microscope (Leica MZ6) a silique was placed in the resin, gently opened and seeds released with the aid of a needle. The pod wall was then discarded. A third coverslip was placed on top so that its edges were supported by the two coverslips already on the slide. After that the slides were moved into an oven at 60°C until the resin polymerized. Slides were then stored at 4 °C in the dark (Braselton *et al.*, 1996).

Confocal laser scanning and bright field microscopy:

The preparation of specimens was carried out according to Braselton *et al.* (1996), and images were taken with an argon ion laser, 488 nm excitation and 515-530 nm emission, using a Nikon C1 confocal microscope system with a 90i Eclipse microscope and EZ-C1 software. All images were captured using 20x lens (Nikon UK). The images were saved as TIFF files then processed using Adobe Photoshop Elements. The sample sizes ranged

between eight to ten seeds derived from different stages of development (either 5, 7 or 9DAP).

Standard bright field microscopy was used to analyse *ban* X Col4x seed at 7 and 9DAP and *anl1* X Col4x seed at 9 DAP by using 10x magnification.

2.2.8 Development of homozygous auxin reporter lines

Seeds carrying the DR5rev::GFP auxin responsive reporter construct in the Col-0 background were ordered from NASC (Scholl *et al.*, 2000). DR5rev::GFP plants were cross-pollinated with *ttg2*, *tt4*, *fls* and *ban* T-DNA insertion lines, followed by recovering homozygous lines carrying DR5rev::GFP/*tt* in the subsequent generations. These plants were then used in diploid and interploidy crosses as described in section 2.2.10 below.

2.2.9 Analysis of homozygous auxin reporter lines

Data for this study were collected using a combination of quantitative and qualitative approaches gained from confocal microscopy. Consequently, DR5rev::GFP mutant line provided an *in vivo* specific proxy for determining auxin response and localisation. Data were collected using confocal microscopy on seeds pollinated by either diploid or tetraploid pollen parents taken at 0 (unfertilised), 3, 5, 7 and 9 days after pollination. The Z stack data was used, along with IMARIS software, for analysis to retrieve GFP fluorescence intensity per μm^3 for the data.

2.2.10 Germination and growth conditions of homozygous auxin reporter lines

DR5rev::GFP/*tt* seed were selected on Sulfonamide-MS plates (see section 2.1.5) in Petri dishes for two weeks. This procedure was carried out until lines were homozygous for both DR5rev::GFP and the *tt* T-DNA insertions (6 generations). For the final experiments involving these lines, sulfonamide resistant large seedlings were selected to be transferred to soil. Meanwhile, seeds for pollen parent (Col4x and Col2x) were placed in 0.1% agar at 4°C for two days. Both sets of seeds were transferred to trays containing Levingtons F2+S (compost + sand) and treated as in section 2.2.1.

2.2.11 Glycol methacrylate embedding for soft tissues of homozygous auxin reporter lines

The samples were prepared in 1.5 ml microfuge tubes using a protocol adapted from that described in 'Technovit H7100 Soft Tissues' (EMS catalog 14653) as described in details below:

Fixation:

A. thaliana siliques (5 and 7 DAP) were cut at both ends by using a razor blade and placed in 1.5 ml microfuge tubes with a freshly prepared fixative (3:1, absolute ethanol: acetic acid) and stored overnight at 4 °C.

Embedding:

At room temperature, the pods were incubated for just two hours each in increasing concentrations of ethanol - once in 70% (v/v) and twice in both 96 % (v/v) ethanol and then 100 % (v/v) ethanol. Pods were transferred to 50:50 100% ethanol and infiltrating solution (100ml of Technovit 7100 resin with 1gm of Hardener I (benzoyl peroxide)) for 2 hours. They were then moved to the infiltrating solution for 2 hours and as previously, the fresh pure infiltrating solution was added before storing overnight at 4 °C. Approximately 3 ml of embedding solution (15 parts of infiltrating solution to 1 part of Hardener II) were added to a mould in a fume hood, and the pods, having been cut into small pieces, were added horizontally and left to set for 2 hours. After 2 hours Technovit 3040 / T227 fast setting resin was used to stick the histoblocks onto the samples block.

2.2.12 Toluidine blue staining for homozygous auxin reporter lines

Toluidine blue (TB) is a simple thiazine metachromatic dye with high affinity for acidic cell components (Sridharan and Shankar, 2012). It stains nucleic acids blue with polyphenolic substances including lignin and tannins. It also stains carboxylated polysaccharide a pinkish purple color (Zander, 2016). Slides, which contain seeds, were washed in a toluidine blue working solution (1g/ml Toluidine blue and 9mg/ml NaCl at pH 2.0~2.5; NovaUltra Special Stain Kits) for four mins. Then washed with tap water three times. Following the slides dried out.

The DPX resinous mounting medium (product number 06522, Sigma-Aldrich) was added before adding the coverslip to fix the slide. After that, the slides were transferred into a safe place at room temperature until the resin polymerized.

2.2.13 Making DR5rev::GFP tetraploids from DR5rev::GFP diploids

Plants treated with colchicine (C9754-100MG from Sigma; stock concentration is 0.1g/ml in ethanol; colchicine is used to duplicate the chromosomal number by inhibiting chromosomal segregation during meiosis) were examined for any size differences in leaf and flower, and compared with the wild- type phenotypic traits.

Diploid DR5rev::GFP seedlings (2 weeks old) were treated with 6µl of 0.1%, 0.3% or 0.5% of colchicine, applied to the plant shoot apex. Inflorescences of the treated plants were screened for the phenotypic variation such as the size of pollen, flowers, and seed sizes to test the success of the colchicine treatment. Seeds were collected from inflorescences that displayed larger flowers, increased pollen and seed sizes. To check that lines were tetraploid they were checked as described in section 2.2.14 (karyotype analysis).

2.2.14 Cytological technique for counting mitotic chromosomes

Young *A. thaliana* flower buds were selected for chromosome squashes.

Fixation: Young buds from relevant plants were immersed in a freshly prepared fixation solution (3 parts absolute ethanol: 1 part glacial acetic acid) and stored at 4°C. Before hydrolysis, fixation was performed for a minimum of 30 minutes.

Processing: The Bailey and Stace (1992) method was used to separate the plant material from the fixative, and it was then hydrolysed in 5N HCl for 10 minutes. The inflorescences were then removed from the HCl and put into freshly prepared 70% ethanol. Following immersion in ethanol, they were dissected under a dissecting microscope. A fine pair of needles and a drop of 45% glacial acetic acid were used in dissection. After dissection, a drop of 1% Aceto-orcein (1g orcein: 55 ml glacial acetic acid: 45 ml distilled H_2O) was added to the dissected buds and covered with a coverslip followed by heating at 60ºC for 4-5 seconds for staining. The samples were then squashed gently, and chromosome count was carried out using a Phase Contrast Microscope (Olympus, BH-2, Japan).

2.2.15 DNA extraction

Genomic DNA was extracted from the leaves of 2-3 week-old plants. DNA extraction was carried out using a protocol suitable for PCR screening of Arabidopsis T-DNA insertion lines. Using a 0.5 ml microfuge tube lid as a cutter, a single area of leaf was removed from a young Arabidopsis plant for DNA extraction. The leaf disk was transferred to a 1.5 ml microfuge tube and homogenised in 100µl DNA extraction buffer (0.14 M d-Sorbitol, 0.22 M Tris - HCL pH 8.0, 0.022 M EDTA pH 8.0, 0.8 M NaCl, 0.8 % CTAB and 0.1 % n- Lauroylsarcosine) using a plastic grinder attached to a drill. Then the sample was incubated at 65°C for 5 minutes; after that 100µl of chloroform was added to the sample. It was then in a vortex for 30 seconds and centrifuged at 16,000*g* for 5 minutes. The clear upper phase was moved to a new 1.5 ml tube, and DNA was precipitated by adding 100µl of isopropanol. The sample was mixed and incubated at room temperature for 15 min. The samples were then centrifuged at 16,000*g* for 20 minutes. After the excess isopropanol had been removed, the DNA pellet was then washed with 1ml of 70% ethanol. The sample was centrifuged at 16,000*g* for 10 minutes. The pellet was left to dry at room temperature. The DNA pellet was then dissolved in 50µl sterile Milli-Q water. The DNA extraction was stored at 4°C or used immediately.

2.2.16 RNA extraction

The RNA was extracted from fresh plant siliques at 7 DAP. Liquid nitrogen was used to freeze siliques immediately following removal from the plant; these were then stored at -80°C until needed. Sterile Pestle and ceramic mortar were used to grind up the frozen siliques to be a powder and RNeasy® Plant Mini Kit was used to isolate the RNA (catalog number 74903-74904, Qiagen, USA) according to the manufacturer's instructions. The RNA concentration was then quantified using a NanoVueTM Plus UV spectrophotometer (GE Healthcare), and equal concentrations of the different RNA samples were obtained afterwards (the sample ranged from 1.8-2.0).

2.2.17 Genotypic analysis of Arabidopsis by amplification of DNA

Following genomic DNA extracted from T-DNA insertion lines and WT Col-0 controls, genotyping was performed using PCR (polymerase chain reaction). Two PCR reactions were used to distinguish T-DNA-containing lines from the wild-type: i) utilising gene-specific primers (flanking the putative insertion site) and ii) a T-DNA specific primer in conjunction with a gene specific primer. PCR products in heterozygous plants carry both DNA bands in gels; however, in a homozygote plant, a DNA band shows only in the T-DNA PCR product; furthermore, a genomic band of the PCR product is displayed in the wild-type plant as illustrated in Figure 2.1.

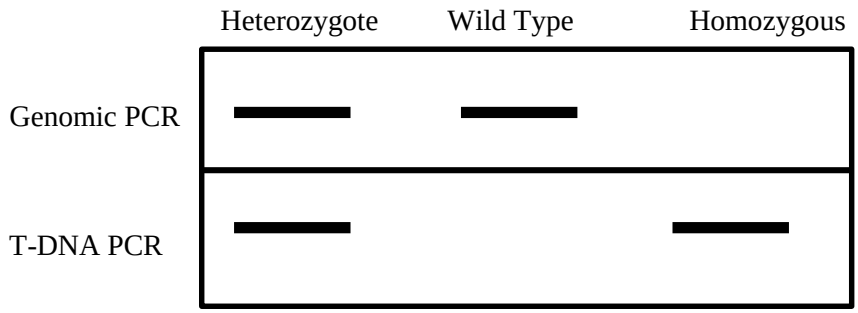


Figure 2.1: The hypothetical results of two PCR gel-loaded products. In a heterozygous sample, both PCR band products are represented, but a band of genomic PCR characterises the wild-type sample, while a homozygous sample is characterised only by a band of the T-DNA PCR.

DreamTaq™ DNA polymerase (product number K1081 from Fermentas) was used to perform PCR. The Dream Taq Green PCR Master Mix (2X) was briefly centrifuged after thawing and was kept on ice. 12.5 µl reaction included: 3.75 µl sterile Milli-Q water, 1 µl of 5 µM forward and reverse primers, 6.25 µl of Taq DNA polymerase and 0.5 µl of DNA template. All reactions were carried out in the PCR machine (PTC-200 Peltier Thermal Cycler, MJ Research, USA). For genotyping plants, a standard amplification programme was used: firstly, an initial denaturation step at 95°C for 2-3 min and subsequently 32 cycles of denaturation at 95 °C for 30 sec, annealing at X°C (where $X = The\ TM\ used\ of\ primer\ pairs - 5℃$), for 30 sec and extension at 72 °C for ~ 1 min (depending on the length of fragment, 1 min/kb), and afterward a final extension for 10 min at 72 °C to finish off the PCR products. Details of the primers can be found in Table 2.3 and Table 2.4. The left primer (LP) and right primer (RP) are always on the 5' end and the 3' end of the insertion, respectively.

Table 2.3: T-DNA insertion lines primers sequence that were used for genotyping.

Transparent testa (tt) mutant	Gene	Primer name	Sequence (5' – 3')	Tm (°C)
tt4-12	At5g13930	GABI_304D03_LP	TACAACAAAGCCCTTTGTTGG	60.02
		GABI_304D03_RP	TACATCATGAGACGCTTGACG	59.88
tt5-3	At3g55120	SALK_034145.51.00.x _LP	CGTTTGTACCGTCCGTCAAG	59.22
		SALK_034145.51.00.x _RP	TGGATATCAAGGCCTCGGAC	58.95
		SALK_034145.51.00.x _LP	GGAGGGAATGTTTCTTCCTTG	59.93
		SALK_034145.51.00.x _RP	TTTTTCTTTGATTTTCCGAAAG	57.63
tt6	At3g51240	SALK_068963.53.50.x_LP	TAAACCCAACGCGTCAAATAC	59.88
		SALK_068963.53.50.x_RP	CTTTACCTCCGGAAGACAAGC	60.25
tt7	At5g07990	SAIL_573_G12_LP	CCGTGTTTTAAAATTTGATAGAGC	58.40
		SAIL_573_G12_RP	AGCTCCGAACGGTATTAGCTC	59.89
fls1	At5g08640	SALK_009992.49.80.x_LP	AGATGATCACTGGTTCGATGC	60.10
		SALK_009992.49.80.x_RP	TATCTGGTGCGGTCTAAGTGC	60.29
tt3	At5g42800	SALK_099848.56.00.x_LP	TTTAGAATCCACGTGGACGAG	60.12
		SALK_099848.56.00.x_RP	ATCACTAGCCATGAACCGATG	59.97
tt11/tt18	At4g22880	SALK_073183.43.00.x_LP	CTGCTTTGAAAGAAGGCACAC	60.04
		SALK_073183.43.00.x_RP	AGCCGGAGAAGAGTTTTTCAG	60.00
tt14/tt19	At5g17220	SALK_105779.45.70.x_LP	CAGGTAACAGCAGCTTGTCC	58.05
		SALK_105779.45.70.x_RP	TATCCGAAATCTCTTCCCACC	60.03
anl1	At5g17050	SALK_049338.56.00.x_LP	CTCTTCGTTATTTTCCTCCGG	60.08
		SALK_049338.56.00.x_RP	TCAAACCCATCTTTCGTGAAG	60.10
ban	At1g61720	SALK_122474.20.75.x_LP	TGTTGCAAGCTAAGGAGTTGG	60.43
		SALK_122474.20.75.x_RP	TATTCACAGCCGGAGAATGAG	60.22
tt12	At3g59030	GABI_797D03 _LP	TGCTTCCAAATGGATCATTTC	59.89
		GABI_797D03 _RP	CAGTGTGGGAGTCAAAGCTTC	59.90
tt10	At5g48100	GABI_146E10_LP	TTTCACCAGTCAAAAATCTTGG	59.11
		GABI_146E10_RP	TCACTAGCTCGGTTTTGAACG	60.43
aha10-4	At1g17260	SALK_019569.47.20.x _LP	CCAACCAAAAGGGAAAGAAAG	59.96
		SALK_019569.47.20.x _RP	AGGGTTTTGACCCTTCTTGAG	59.60
ttg2	At2g37260	SALK_148838.51.70.x_LP	TAAAACCAAACGACACCGTTC	59.89
		SALK_148838.51.70.x_RP	TCCAAGTTTGTTGACGATTCC	59.96
		GAPC-LP	CACTTGAAGGGTGGTGCCAAG	62.7
		GAPC-RP	CCTGTTGTCGCCAACGAAGTC	62.8
SALK left border primer		LBb1.3	ATTTTGCCGATTTCGGAAC	
GABI left border primer		GABI-Kat o8409	ATATTGACCATCATACTCATTGC	
SAIL left border primer		LB1 SAIL	GCCTTTTCAGAAATGGATAAATAG CCTTGCTTCC	

Table 2.4: PCR conditions used for genotyping.

tt mutants	Seed stock number	Primer name	Annealing tm (°C)	Fragment length (bp)
tt4-12	N306597	GABI_304D03_LP	55.02	
		GABI_304D03_RP	54.88	446-746
tt5-3	N534145	SALK_034145.51.00.x _LP	55.04	
		SALK_034145.51.00.x _RP	55.02	544-844
tt6	At3g51240	SALK_068963.53.50.x_LP	54.88	
		SALK_068963.53.50.x_RP	55.25	604-904
tt7	N568963	SAIL_573_G12_LP	53.4	
		SAIL_573_G12_RP	54.89	541-841
fls1	N824443	SALK_009992.49.80.x_LP	55.10	
		SALK_009992.49.80.x_RP	55.29	536-836
tt3	N599848	SALK_099848.56.00.x_LP	55.12	
		SALK_099848.56.00.x_RP	54.97	436-736
tt11/tt18	N573183	SALK_073183.43.00.x_LP	55.04	
		SALK_073183.43.00.x_RP	55	456-756
tt14/tt19	N605779	SALK_105779.45.70.x_LP	53.05	
		SALK_105779.45.70.x_RP	55.03	582-882

anl1	N25064	SALK_049338.56.00.x_LP	55.08	
		SALK_049338.56.00.x_RP	55.10	581-881
ban	N622474	SALK_122474.20.75.x_LP	55.43	
		SALK_122474.20.75.x_RP	55.22	560-860
tt12	N339468	GABI_797D03 _LP	54.89	
		GABI_797D03 _RP	54.90	559-859
tt10	N395787	GABI_146E10_LP	54.11	
		GABI_146E10_RP	55.43	460-760
aha10-4	N519569	SALK_019569.47.20.x _LP	54.96	
		SALK_019569.47.20.x _RP	54.60	490-790
ttg2	N648838	SALK_148838.51.70.x_LP	54.89	
		SALK_148838.51.70.x_RP	54.96	572-872
		GAPC-LP	57.80	
		GAPC-RP	57.80	
SALK left border primer		LBb1.3		
GABI left border primer		GABI-Kat o8409		
SAIL left border primer		LB1 SAIL		

2.2.18 cDNA synthesis

cDNA was synthesised from RNA using the ThermoScript™ RT-PCR System (catalog no.11146-024 from Invitrogen™) following the manufacturer's instructions. Then PCR was performed with the cDNA as a template (2.2.17). Primers details are available in Table 2.5.

Table 2.5: RT- PCR primers.

***tt* mutants**	**Gene**	**Primer name**	**Sequence (5' – 3')**	**Product size (BP)**	**Tm (°C)**
tt4-12	At5g13930	GABI_304D03_LP	ACATGACCGACCTCAAGGAG	566	60.11
		GABI_304D03_RP	GGGTTTCTCTCCGACAGATG		59.65
tt5-3	At3g55120	SALK_034145.51.00.x _LP	CGTTTGTACCGTCCGTCAAG	426	59.22
		SALK_034145.51.00.x _RP	GGGAGAGAGCGAAGAGGATC		59.04
tt6	At3g51240	SALK_068963.53.50.x_LP	CGTCAGATCGTTGAGGCTTG	471	59.01
		SALK_068963.53.50.x_RP	GAGTCCGAGGGTGAGATCAG		58.97
		SALK_068963.53.50.x_LP	TCTTACCAATGCATGCGTCG	491	58.99
		SALK_068963.53.50.x_RP	CTTCTTTGTGGTCACGCTCC		59.13
tt7	At5g07990	SAIL_573_G12_LP	GGTGACGGAAATGATGGCTC	485	58.98
		SAIL_573_G12_RP	GTGTTGGTGGATGAAGCCTG		59.12
		SAIL_573_G12_LP	GCACTAAGCCTCATCGAACC	490	59.84
		SAIL_573_G12_RP	AGAGCCATCATTTCCGTCAC		60.08
fls1	At5g08640	SALK_009992.49.80.x_LP	GGTGGTGAAAGCGAGTGAAG	593	59.13
		SALK_009992.49.80.x_RP	TGACGGCGGAGGGAATATAC		59.04
tt3	At5g42800	SALK_099848.56.00.x_LP	AAATCCCACGCTGCTTTCTC	444	58.83
		SALK_099848.56.00.x_RP	TTACTTTGTTCGTGCCACCG		59.06
tt11/tt18	At4g22880	SALK_073183.43.00.x_LP	AAACCCGGAACCATGTTGTG	471	58.96
		SALK_073183.43.00.x_RP	ATGGAGCGTGTCAAGAAAGC		58.84
tt14/tt19	At5g17220	SALK_105779.45.70.x_LP	GCAGCTTGTCCACAAAGAGTC	435	60
		SALK_105779.45.70.x_RP	CCAAGACGGTCTATCCGAAA		60.01
anl1	At5g17050	SALK_049338.56.00.x_LP	TGACTGATGCGTTCTTCTGG	548	60
		SALK_049338.56.00.x_RP	TTCCAACCCTTCTGCTATCG		60.2

ban	At1g61720	SALK_122474.20.75.x_LP	ATCTTCCATGTCGCAACTCC	510	60.01
		SALK_122474.20.75.x_RP	AAGAAACAAATGGGCACGAG		60.01
		SALK_122474.20.75.x_LP	GAACCGGAATCGTGATGAAC	412	60.3
		SALK_122474.20.75.x_RP	ATCCGCAATCTCTGGAACAC		60.1
tt12	At3g59030	GABI_797D03 _LP	CGTCATGTTCACCGGTCATC	582	59.00
		GABI_797D03 _RP	AATGCCCTCGTTGAAAACCC		59.03
		GABI_797D03 _LP	GACAGGGTTTTCAACGAGGG	341	58.76
		GABI_797D03 _RP	ACACAAGCACGATGACACAG		58.78
tt10	At5g48100	GABI_146E10_LP	AGTGGTGGAAGAGGGATGTG	190	59.01
		GABI_146E10_RP	ATTGCGGCATTTACCATCCG		59.33
aha10-4	At1g17260	SALK_019569.47.20.x _LP	TGTGAATGATGCTCCTGCTC	347	60
		SALK_019569.47.20.x _RP	GGTTGAGCTTCCAACTCTCG		60
ttg2-3	At2g37260	SALK_148838.51.70.x_LP	ATGCACACACCCGAAATGTC	470	59.12
		SALK_148838.51.70.x_RP	CTGATTCCACTGAGCCTTGC		58.91
		GAPC-LP	CACTTGAAGGGTGGTGCCAAG	542-812	
		GAPC-RP	CCTGTTGTCGCCAACGAAGTC		

2.2.19 Agarose gel electrophoresis

The products of PCR were run on a 1% agarose gel. This gel was made using electrophoresis grade agarose (Fisher, UK) dissolved in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA, 1.14% glacial acetic acid, pH 7.6) by gently heating in a microwave oven; when the molten agar had cooled to ~60ºC, Ethidium bromide was added to a final concentration of 0.2 μg/ml (10 mg/ml Sigma). The gel was left to set for 30-60 min, and then submerged in 1x TAE buffer in a gel Sub-Cell tank (Bio-Rad). A 100bp DNA ladder (1 μg/μl, Cat. No. 15628-019 from Invitrogen**)** was loaded onto each gel as a molecular weight marker. Electrophoresis was carried out using a PowerPC 300 power supply (Bio-Rad) at ~ 100 V for 25 minutes. The DNA bands were visualised on a UV transilluminator (BioDoc).

Chapter 3: Impact of maternal FBP mutations on the triploid block in a range of *A. thaliana* ecotypes

3.1 Introduction

It is now well established that interaction between the endosperm and integuments during seed development is an important determining factor of the final size of seeds (Garcia *et al.*, 2003; Garcia *et al.*, 2005; Luo *et al.*, 2005; Berger *et al.*, 2006; Locascio *et al.*, 2014; Doughty *et al.*, 2014). Thus, aberrant development of either zygotic (embryo and endosperm) or maternal tissues (seed coat) has the potential to impact on seed size. Frequently endosperm development can be unbalanced by interspecific and interploidy hybridization and seeds resulting from such crosses show striking size variation (Scott *et al.*, 1998). There is now evidence that the aberrant endosperm development characteristic of such crosses can be influenced by maternal modifier genes. Importantly a number of genes involved with the operation of the flavonoid biosynthesis pathway (FBP) can act as such maternal modifiers, and these can re-impose a normal (balanced) programme of endosperm development restoring –more-or-less normal viability.

3.1.1 Effect of interploidy crosses and parent-of-origin effects on seed size and viability

In crop plants, the growth of the endosperm directly affects final seed size. Moreover, during early seed development, endosperm growth is correlated with testa growth. This could have an important role in deciding how big the embryo sac is after fertilisation that, in turn, affects the size of both the embryo and seed (Scott *et al.*, 1998; Bradford *et al.*, 2007). Thus, final seed size can be modified by manipulation of endosperm growth, which is itself influenced by parent-of-origin effects in interploidy crosses (Scott *et al.*, 1998; Bradford *et al.*, 2007). For instance, in interploidy crosses between diploid maternal parental lines and pollen parents of higher ploidy (e.g. pollen derived from 4x or 6x plants), there is an imbalance in the contribution of each of the parental genomes, which affects endosperm growth and development. This is characterised by over-proliferation of endosperm nuclei, delayed endosperm cellularisation, and overgrowth of the chalazal endosperm (Scott *et al.*, 1998). The latter leads to increased transference of nutrient resources from the mother plant producing a large embryo and endosperm, thereby resulting in an increase in seed size in comparison to progeny from diploid

parents (Scott *et al.*, 1998; Moise *et al.*, 2005; Bradford *et al.*, 2007). Conversely, small seeds result from a reciprocal cross of the previous example where maternal ploidy is higher (e.g. 4x or 6x) than that of the pollen parent. Such crosses lead to an inhibition of endosperm growth (due to under-proliferation of endosperm nuclei) and precocious (early) endosperm cellularisation that results in a small embryo and endosperm[2] (Scott *et al.*, 1998).

For most *Arabidopsis thaliana* ecotypes, seed development following interploidy crosses between maternal diploid (2x) and paternal tetraploid plants (4x), results in large, viable seeds. However, in such crosses involving the Col-0 ecotype, the higher paternal genomic contribution in the endosperm negatively affects seed development leading to the production of inviable seeds. This is commonly referred to as the triploid block - thus any crosses with pollen parents having a ploidy higher than 2x would render the seeds inviable (Scott *et al.*, 1998; Dilkes *et al.*, 2008). The observed variation in the outcome of such interploidy crosses amongst *A. thaliana* ecotypes clearly points to the fact that there is a genetic basis for modification of the triploid block and raises the important question of which genes are responsible.

3.1.2 The FBP and genetic regulation of seed development

As mentioned previously, there are three seed components; two of them are zygotic tissues (embryo and endosperm) and one is solely of maternal origin (testa) (Sundaresan, 2005) and interaction between them is important in determining seed size – understanding the roles of these different domains has been a focus of research in recent years. In *A. thaliana,* the division of cells that form the integument and their differentiation into the seed integument (testa) ends by about four days after pollination (DAP). Following this initial differentiation phase, cell elongation continues until approximately 6-7 DAP, which is the duration it takes for the seeds to reach their maximum length (Alonso-Blanco *et al.*, 1999; Garcia *et al.*, 2005). It is considered that final seed size and extent of endosperm growth may be determined by the size and number of cells in the testa (Garcia *et al.*, 2005).

[2] For more details please refer to section 1.5 in Chapter1.

Investigating the molecular genetic basis of seed size has reinforced the thesis that the testa plays a crucial regulatory role. For example, mutants of TTG2[3] (an important transcriptional regulator of the flavonoid biosynthesis pathway), as well as having pale seeds due to loss of flavonoid pigments in the seed coat, also have reduced cell elongation in the integuments (Johnson *et al.*, 2002; Garcia *et al.*, 2005; Bradford *et al.*, 2007). Moreover, Garcia *et al.* (2005) demonstrated that the size of the *A. thaliana* seed is affected by the maternal version of *ttg2* (Johnson *et al.*, 2002; Garcia *et al.*, 2005; Bradford *et al.*, 2007) (see sections 1.6.1 and 1.9.3). Thus, although *TTG2* appears to be an important developmental regulator with respect to cell elongation in the integuments, intriguingly it raises the question as to whether components of the FBP may also have an important role to play in shaping seed development.

3.1.3 Relationship between interploidy hybridisation, triploid block and the FBP

Currently, explanation of the triploid block in Col-0 seeds is that cellularisation of the endosperm is influenced by a factor that could move between the integument layers and the endosperm (Dilkes *et al.*, 2008). Moreover, previous work by Dilkes *et al.* (2008) has demonstrated that seeds derived from crosses between *ttg2* maternal plants (2x) and Col4x (tetraploid Col-0) pollen parents are viable (*ttg2* overcomes the triploid block). As proposed in the model described in section 1.7, the accumulation of FBP products in the seed integuments layer could repress a maternal cellularisation factor, this would inhibit cellularisation of the endosperm, and seeds would die, as cellularisation of the endosperm would not occur appropriately in Col 2x X 4x crosses. Another an evidence for an involvement of the FBP in regulating seed development comes from analysis of *tt* class maternal mutations, which typically affect enzymatic steps of the FBP. These mutations cause defects in the seed coat, which in turn influence seed mass (Debeaujon *et al.*, 2000).

Importantly, previous preliminary work in Scott Laboratory (University of Bath) looked at the effect of mutations in the FBP on the outcome of paternal excess (Col4x) interploidy crosses when the mutations were carried by the 2x mother. This work showed that the FBP had a role in influencing the timing of endosperm cellularisation, as lethal

[3] A transcription factor involved in regulation of the Flavonoid Biosynthesis Pathway (FBP) and is weakly expressed in the endosperm but strongly expressed in the seed testa especially the endothelium layer (Johnson *et al.*, 2002; Garcia *et al.*, 2005).

2x X 4x crosses were rescued when the mother harboured an FBP mutation. However, unfortunately, the FBP mutations studied were carried in a range of *A. thaliana* ecotypes and thus comparisons between them were complicated by the ecotypic variation for seed development. Thus to fully understand the role of the FBP and its intermediates in the regulation of seed development a single ecotype study would be of great value.

3.1.4 Aims and objectives of the chapter

Work presented in this chapter set out to confirm the preliminary work carried out by the Scott laboratory that maternal mutations in the FBP could rescue paternal Col 4x induced seed lethality in 2x X 4x crosses. This pilot study utilised Col-0, L*er* and WS ecotypes in interploidy crosses. Additionally, a thorough single ecotype (Col-0) survey on the effect of maternal FBP mutations on seed development in 2x X 4x crosses was carried out. This focussed study would eliminate concerns over inter-ecotype variation for the role of the FBP in rescuing Col4x induced seed lethality. T-DNA insertion lines for genes encoding all enzymes involved in the FBP along with two regulatory factors (TTG2 and TT16) were included in the study.

3.2 Results

The data presented in the following sections will serve to study the relationship between the timing of the endosperm cellularisation and the ability of maternal mutations in the FBP to rescue Col4x induced seed lethality. This will shed light on the theory that product(s) of the FBP, synthesised in the endothelium may titre a cellularisation factor that moves between the seed coat and the endosperm.

3.2.1 Seed development following interploidy crosses involving maternal FBP mutants in different *A. thaliana* ecotypes

In this section, results are presented from a preliminary study that utilised a small range of ecotypes (Col-0, L*er*, and Ws) carrying *tt* mutants (as seed parents) in interploidy crosses with Col 4x pollen parents. In order to assess whether mutation of genes in the FBP could lead to rescue of Col-0 4x-induced lethality in these ecotypes data for the stage

of embryo development, degree of endosperm cellularisation, maximum cross-sectional area of the embryo sac, chalazal area, number of endosperm nodules, dry seed weight and mature seed phenotypes was collected.. The FBP mutants studied were *fls-1* (Col-0 ecotype); *tt4-1*, *ttg2-1* and *tt10-5* (L*er* ecotype); and *tt12-1* and *tt16* in the Ws ecotype. Seeds were compared to their controls at different time points (5 and 7 DAP or 5 and 9 DAP) to assess effects on embryo and endosperm development.

3.2.1.1. Previously obtained data

The balanced cross data (2x X 2x) is presented in figure 3.1. At 5 DAP, in the micropylar peripheral endosperm, the suspensor is normally sunk. The chalazal endosperm has a dominant multinucleate tissue and central peripheral endosperm lines the central region of the embryo sac. The peripheral endosperm is cellularised and the embryo has reached the heart stage. By 7 DAP, and while the chalazal endosperm is still present, the embryo reaches the torpedo stage with the appearance of compressed micropylar peripheral endosperm. At 9 DAP, the chalazal endosperm is lost and the seed is almost completely filled by the embryo. By seed maturity, such balanced crosses generate viable, plump seeds (Figure 3.1).

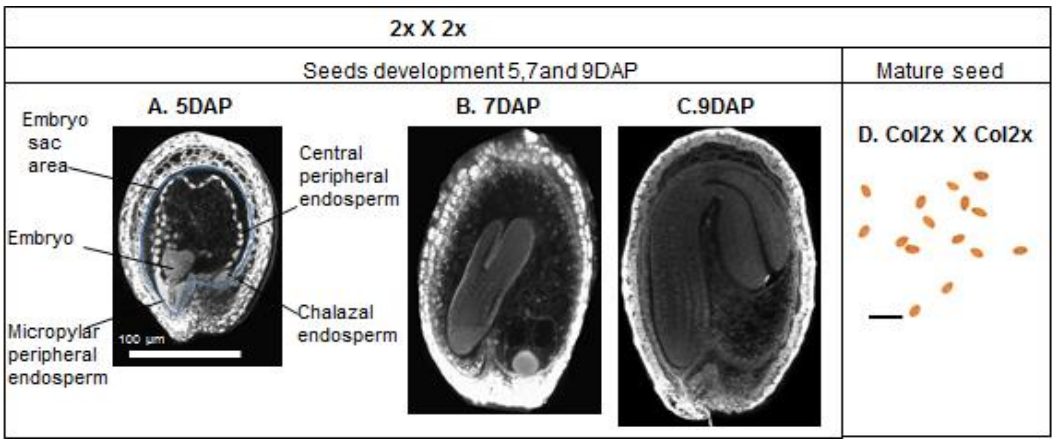


Figure 3.1: Seed development following diploid crosses (2x X 2x) in the Col-0 ecotype of *A. thalia*na. The development of an embryo and chalazal endosperm as demonstrated by confocal micrographs of Feulgen-stained seeds: **A.** 5 DAP is displayed - the micropylar (MP) and chalazal poles (CP), respectively, **B.** 7 DAP stage, and **C.** 9 DAP stage showing the cotyledons of the now well-developed embryo. All confocal images were taken at 20x magnification. Scale bars, 100µm. **D.** Mature seeds image shows the typical plump seed phenotype typical of diploid Col-0 wild-type crosses. Scale bars, 1 mm.

3.2.1.2. A. thaliana ecotypes behave differently during seed development following interploidy crosses to Col4x pollen parents

Compared to balanced crosses (Figure 3.1) 2x X 4x crosses in all ecotypes display a paternal excess phenotype with markedly retarded embryo development. At 5 DAP, the seed derived from 2x X 4x Col-0 parents had an early heart stage embryo, whilst the seed from the L*er* parents was still globular and appeared smaller but with better-defined structures than the seed with two Col-0 parents (Figure 3.2.A and B). By 7 DAP embryos developed to the early torpedo stage in the L*er* 2x X Col4x crosses whereas seeds from Col2x X Col4x crosses had lost a recognisable embryo and had ill-defined internal morphology. A greatly thickened seed coat was also present in seed resulting from the Col2x X Col4x cross along with a very prominent chalazal endosperm, (Figure 3.2.B), whereas the seeds resulting from L*er*2x X Col4x crosses were generally smaller and had many defined structures by 7 DAP; both these features are likely to be due to the L*er* ecotype influence (early endosperm cellularisation) (Dilkes *et al.*, 2008). In the L*er*2x X Col4x cross, the chalazal endosperm is often enlarged, and vacuolated (Figure 3.2.D). The proliferation of the endosperm still occurs around 5 DAP in the L*er* 2x X Col 4x cross, similar to the case for Col2x balanced crosses with cellularisation being evident. However, the Col2x X Col4x seeds, which typically display 80% lethality, often did not show any significant endosperm cellularisation. Interestingly, the size of chalazal endosperm in the Col2x X Col4x cross was larger than that observed in Col2x X Col2x crosses (Figure 3.1).

Furthermore, to the seed phenotypes described above the size and shape of seeds resulting from 2x X Col4x crosses varied depending on the ecotype of the seed parent. For example, seeds generated by Col-0 plants were longer and more oval than the other ecotypes whilst seeds derived from L*er* mothers were rounder than those from the other ecotypes studied (Figure 3.2). This limited pilot study confirms that there is significant ecotype variation in *A. thaliana* for developmental responses to paternal excess in 2x X 4x crosses.

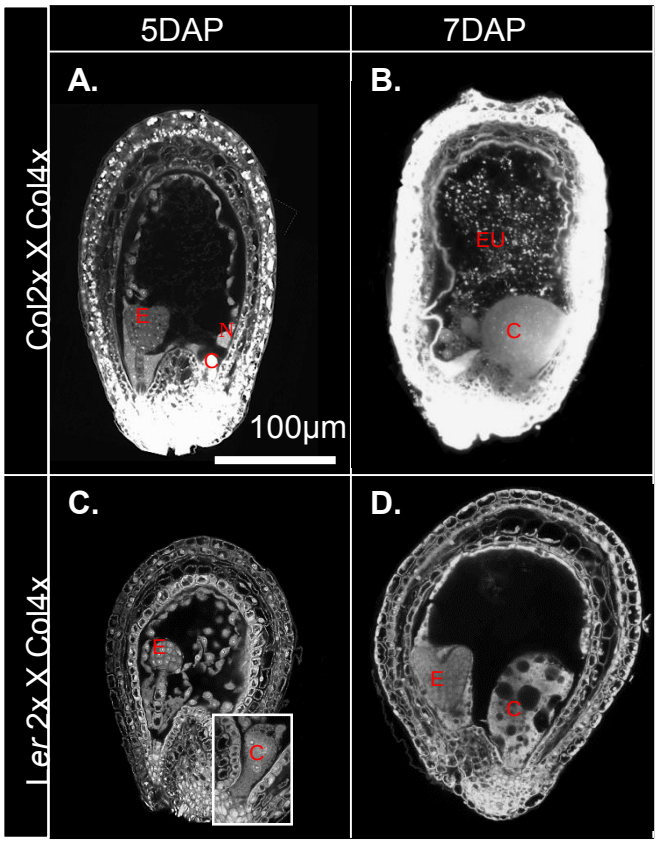


Figure 3.2: Confocal laser scanning photomicrographs of developing seeds derived from Col2x and L*er* 2x ecotypes crossed with Col4x-pollen parents All images were taken at 20x magnification and two developmental time points are presented, 5 and 7 days after pollination (DAP). **E**, embryo; **C**, chalazal endosperm; **N**, nodules; **EU**, endosperm un-cellularised. Scale bar = 100µm in all images.

3.2.1.3. Interploidy crosses between several FBP maternal mutant lines from different ecotypes and a paternal excess (Col4x) line, have significant and varied effects on seed development

3.2.1.3.1. Cross between maternal *fls-1* and paternal Col4x

Results showed that seeds derived from crosses between maternal *fls-1* and paternal Col4x plants were reduced in size and contained embryos that were poorly defined (Figure 3.3). FLS is an enzyme responsible for the synthesis of flavonols.

Seeds derived from the control cross (Col2x X Col4x) typically showed a severe delay in progression to the heart stage of embryo development (Figure 3.3.A, B and C), whereas the embryos of *fls-1* x Col4x crosses were never observed to reach the heart stage with development being arrested at the globular stage at 5 and 7 DAP (Figure 3.3.D and 3.3.E) and late globular stage at 9 DAP (Figure 3.3.F). This suggests that mutations of genes for the FBP can strongly influence seed development.

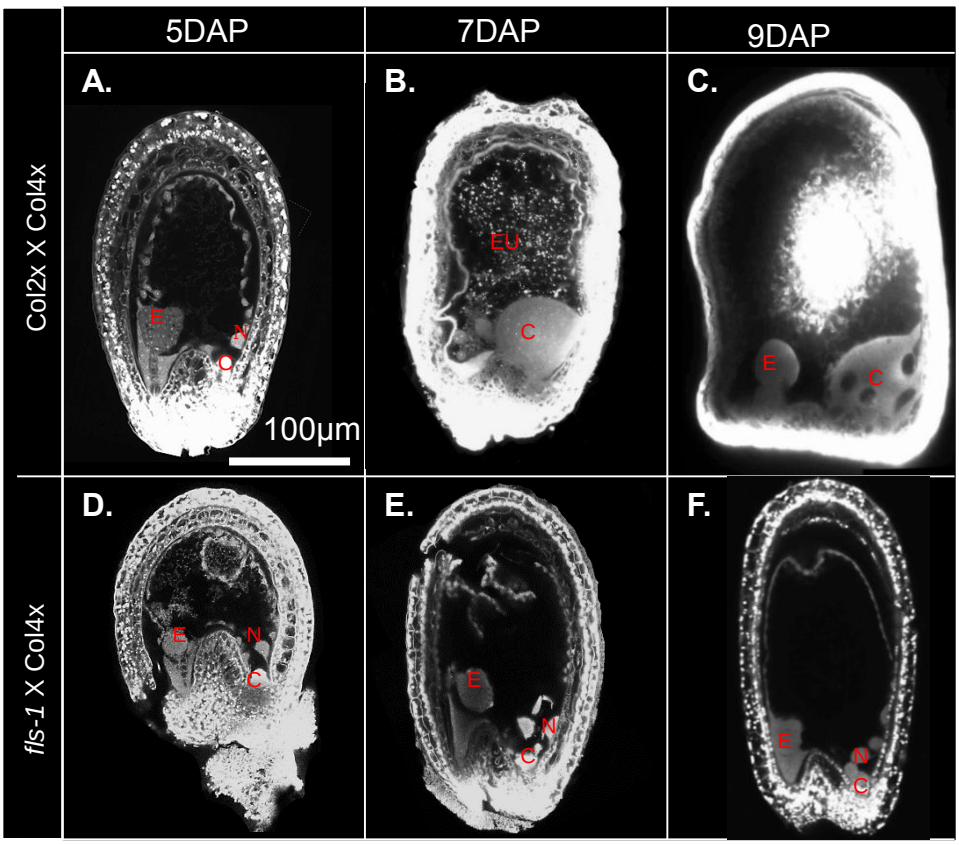


Figure 3.3: Confocal laser scanning photomicrographs of developing seeds descending from a cross between maternal *fls-1* plants in the Col-0 background with paternal Col4x, at 5, 7 and 9 DAP. All images were taken at 20x magnification. **E**, embryo; **C**, chalazal endosperm; **N**, nodules; **EU**, endosperm un-cellularised. Scale bar = 100µm in all images.

3.2.1.3.2. FBP mutations in the L*er* ecotype influence seed lethality in interploidy crosses

Seeds derived from a cross between *tt4-1* in L*er* ecotype with Col4x reached the heart stage of embryo development by 5DAP (Figure 3.4.C) and attained the late heart stage by 7DAP. Moreover, the seeds of *tt4-1* X Col4x showed signs of endosperm cellularisation at 5DAP (Figure 3.4.C) whilst endosperm proliferation was still occurring in the seeds of the control L*er*2x X Col4x at this stage (Figure 3.4.A). By 7DAP, seeds from L*er*2x X Col4x crosses had a very large and vacuolated chalazal endosperm, whereas *tt4-1* X Col4x seeds had a much smaller chalazal endosperm by 7DAP. These characteristics suggest that the *tt4* mutation on the maternal side ameliorates the effects of paternal Col4x induced seed lethality.

In a similar manner to the observations for *tt4* seed parents, *ttg2*-1 (L*er* background) X Col4x seeds were found to reach the heart stage of embryo development by 5DAP with some evidence of endosperm cellularisation taking place (Figure 3.4.E). By 7DAP, the early torpedo stage was reached (Figure 3.4.F).

In contrast, *tt10*-5 (L*er* background) X Col4x crosses resulted in seeds carrying embryos at the globular stage by 5DAP (Figure 3.4.G) that failed to progress normal development beyond this point producing an abnormal globular-like embryo by 7DAP, (Figure 3.4.H). Cellularisation of endosperm was also delayed in these seeds in comparison to the control L*er*2x X Col4x cross (at 7DAP).

In summary, both *ttg2* and *tt4* mutations present in the seed parents of crosses to Col4x pollen parents resulted in seeds that progressed relatively normally through development being characterised by accelerated cellularisation of endosperm compared to the control L*er* 2x X Col4x crosses. In contrast, the *tt10* mutation appears to have a negative impact on development with serious perturbation of embryo development by 7DAP.

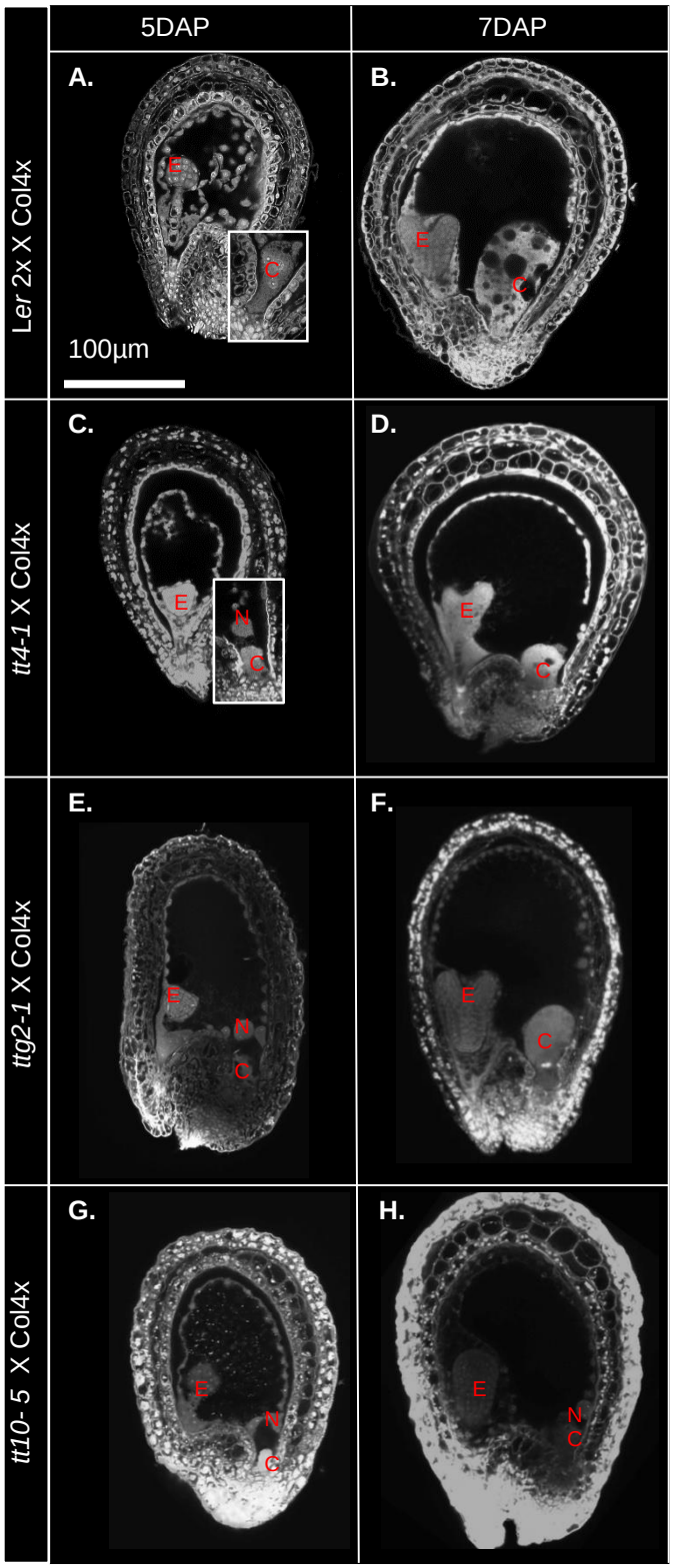


Figure 3.4: Confocal laser-scanning photomicrographs of developing seeds derived from crosses between FBP mutants (in the L*er* ecotype) and Col4x pollen parents at 5 and 7DAP. All confocal images were taken at 20x magnification. **E**, embryo; **C,** chalazal endosperm; **N**, nodules; **EU**, endosperm un-cellularised. The white box in (**A**) and (**C**) show chalazal endosperm of L*er*2x X Col4x and *tt4-1 X* Col4x respectively at 5DAP. Scale bar = 100µm in all images.

3.2.1.4. Paternal excess interploidy crosses are correlated with an increase in embryo sac area across ecotypes and a range of FBP mutant seed parents

Embryo sac area was measured in seeds derived from a range of crosses between FBP mutant seed parents and Col4x pollen parents at 5, 7 and 9 DAP. Embryo sac area was generally seen to increase in all ecotypes in interploidy crosses studied (Figure 3.5). This preliminary data indicated that L*er*2x X Col4x seeds had the largest embryo sac area at 5DAP, with a mean of 32167 µm^2, whereas Col2x X Col4x seeds had the smallest embryo sac area at 5DAP, with a mean value of 6264 µm^2. Though by 7DAP this trend was reversed with Col2x X Col4x seeds having the greatest embryo sac area having a mean value of 58738 µm^2. Conversely, *tt10-5* X Col4x seeds had the lowest embryo sac area at 7DAP, with a mean value of 32345 µm^2.

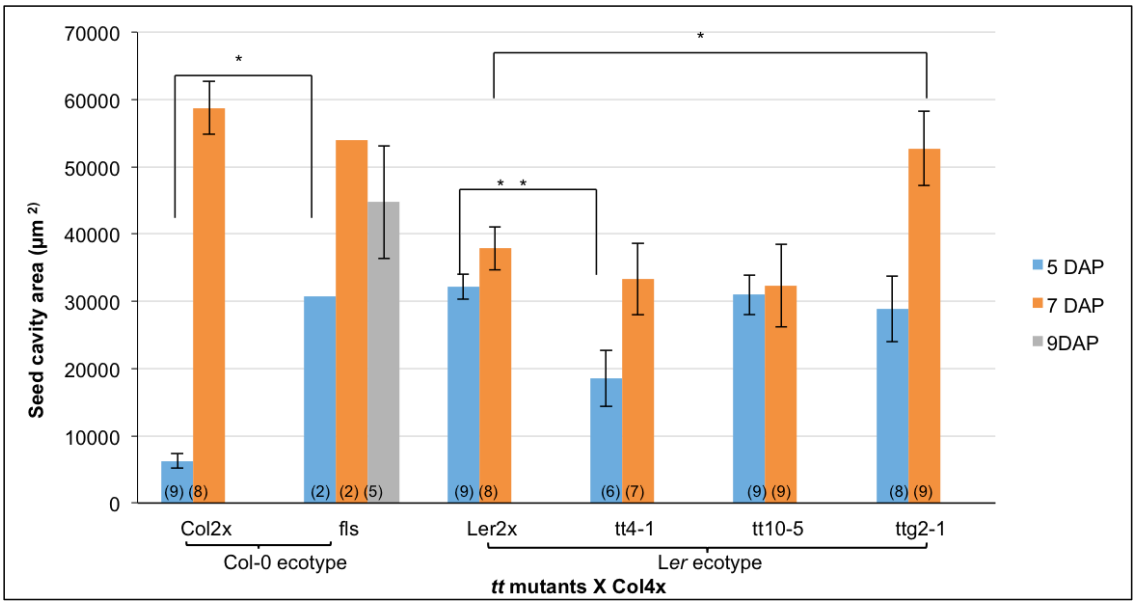


Figure 3.5: Mean embryo sac areas for seeds resulting from the cross between *tt* mutants (in different backgrounds) with Col4x at various stages of development. Embryo sac areas are represented using blue, orange and grey bars to show 5, 7 and 9 DAP respectively. Mean areas were measured in µm^2, being estimated from confocal micrograph images captured at 20x magnification. Error bars represent standard error. Sample sizes (**n**) are in brackets that indicate seeds number. Seeds were collected from two siliques. Significant differences were shown by a Mann-Whitney test: *, P ≤ 0.05; **, P ≤ 0.01.

3.2.1.5. Crosses between FBP mutant seed parents and Col 4x pollen parents leads to increases in chalazal endosperm area

In this pilot study seeds derived from *fls-1*, L*er*2x and *tt4-1* seed parents crossed to a Col4x pollen parents, chalazal endosperm areas approximately doubled between 5 and 7DAP (Figure 3.6). Moreover, Col2x and *ttg2-1* crosses to Col4x result in seeds having an increase in chalazal endosperm size of about 6-fold from 5 to 7DAP. However, the chalazal endosperm area in *fls-1* (Col-0) X Col4x seed at 9DAP is reduced to about half the chalazal endosperm area at 7DAP (see Figure 3.6).

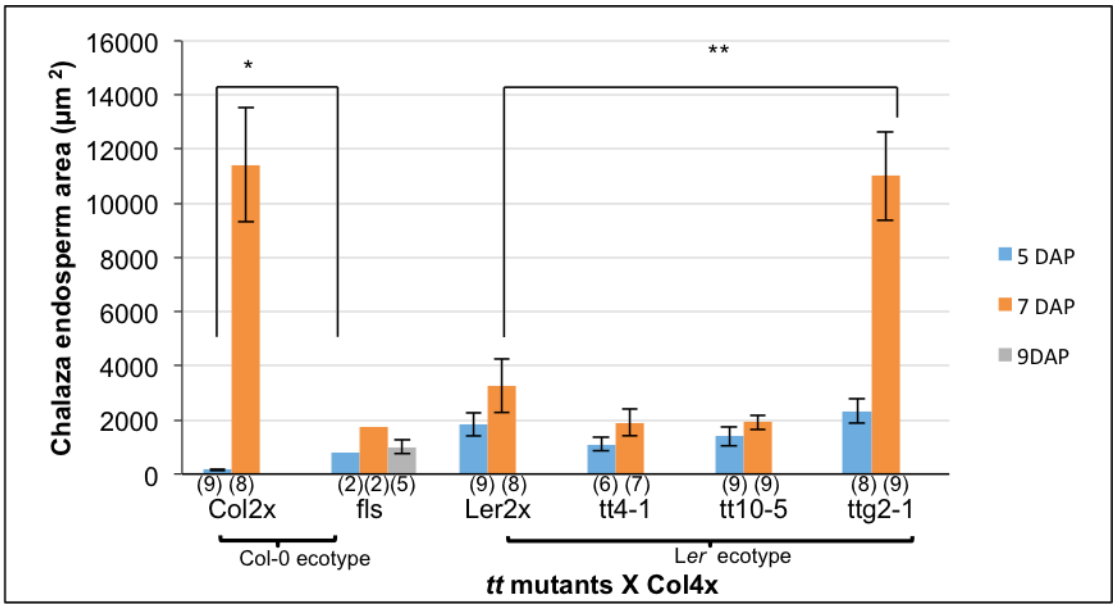


Figure 3.6: Mean value of the area of chalazal endosperm in *tt* mutant seeds resulting from crosses to Col4x pollen parents. Seeds are represented using blue, orange and grey bars to show 5, 7 and 9 DAP, respectively. Mean areas were measured in µm^2, being estimated from confocal micrograph images captured at 20x magnification. Error bars represent standard error. Sample sizes (**n**) are in brackets that indicate seeds number. Seeds were collected from two siliques. Significant differences were shown by a Mann-Whitney test: *, $P \leq 0.05$; **, $P \leq 0.01$.

3.2.1.6. The number of endosperm nodules is influenced by maternal ecotype and mutations in the FBP in the seeds resulting from crosses to Col 4x pollen parents

The average number of nodules was counted at 5 and 7DAP for different crosses (Figure 3.7). At 5DAP, all seeds resulting from crosses involving maternal FBP mutants had lower numbers of nodules than their controls. At 7DAP, the average number of nodules was the highest in Col2x X Col4x, with a mean of 2, this showing an increasing trend from 5 DAP. In crosses involving the L*er* background seed parents harbouring *tt4-1* and *ttg2-1* mutations had more nodules, with the mean of 1.2 and 1, respectively compared to

the control L*er*2x X Col4x cross but still lower than that seen in the Col 2x X Col 4x cross. In Col2x X Col4x, *tt4-1* X Col4x and *ttg2-1* X Col4x seeds, the average number of nodules was larger at 7DAP than at 5 DAP. However, in the other crosses such as L*er* X Col4x and *tt10-5* X Col4x, the average numbers of nodules were lower in 7DAP than 5DAP.

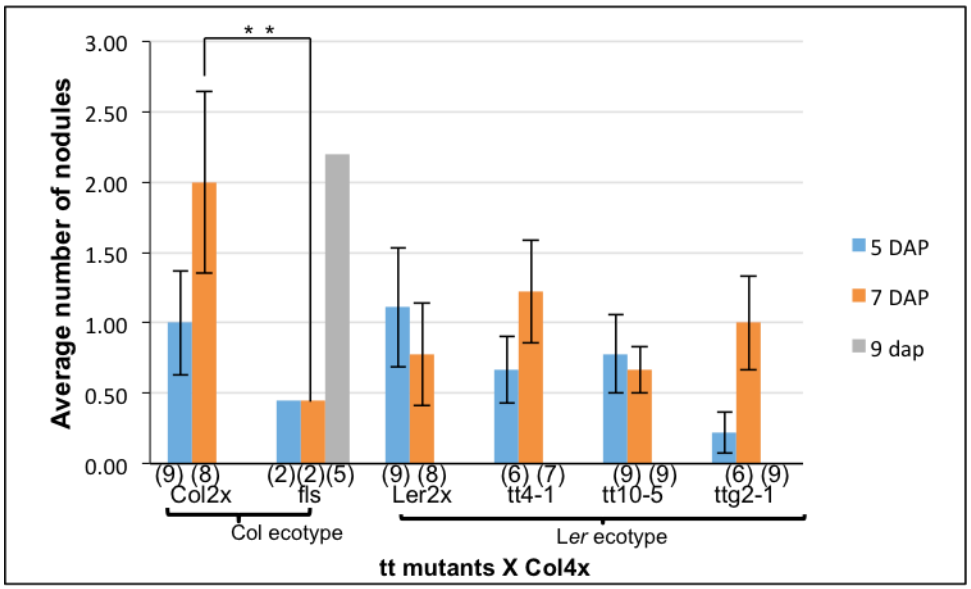


Figure 3.7: Average number of endosperm nodules in seeds resulting from crosses between FBP mutant seed parents and Col 4x pollen parents. Blue, orange and grey bars represent data from seeds at 5, 7 and 9DAP, respectively. The mean number of nodules was estimated from confocal micrograph images captured at 20x magnification. Error bars represent standard error. Sample sizes (**n**) are in brackets that indicate seeds number. Seeds were collected from two siliques. Significant differences were shown by a Mann-Whitney test: **, $P \leq 0.01$.

3.2.2 Mature seed phenotype is influenced by both ecotype and maternally carried mutations of the FBP in crosses to Col 4x pollen parents

3.2.2.1 Effect on seed weight

The weight of mature dry seeds derived from crosses of maternal FBP mutants in a range of ecotypes with Col4x pollen was measured in this pilot study for *fls-1, tt4-1, ttg2-1, tt10-5, tt12-1* and *tt16* plants. It was shown that most *tt* mutants exhibited significant differences in seed weight compared to their respective controls (crosses using the appropriate maternal background ecotypes to Col4x: Col2x X Col4x, L*er*2x X Col4x and Ws X Col4x) (Figure 3.8). Seeds derived from crosses involving *ttg2-1*, L*er*, *tt12-1* and *tt4-1* with the Col4x pollen parent resulted in high mean seed weights. In contrast *fls-1* X Col4x cross-generated seeds were barely measurable being exceedingly light. These seeds were structurally very weak and difficult to handle and weigh because seed weight

value was likely to be very low, -indeed lower than the lowest accurate value- (0.0000001 g).

Other *tt* mutants also generated low mean seed weights in crosses with Col4x, namely *tt16* and *tt10-5*. These mutants did not rescue the abnormal Col4x-induced seed development observed for Col2x X Col4x crosses.

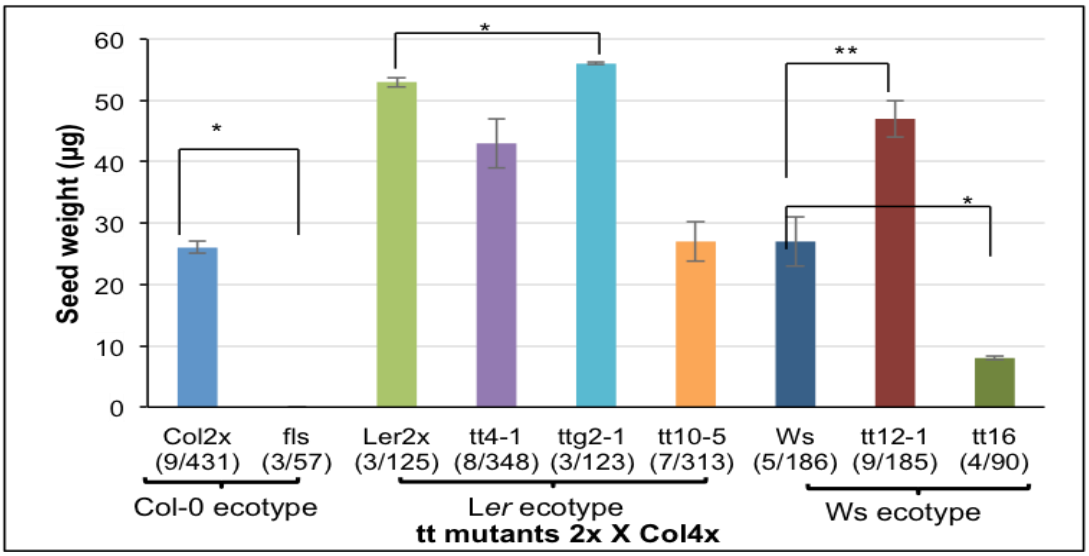


Figure 3.8: The average weights of seeds derived from crosses between FBP mutant seed parents (in different *A. thaliana* ecotypes) and Col 4x pollen parents. The weight of seeds was measured in **µg**. Error bars represent standard error. (**n/n**): the number of siliques /and total numbers of seeds for each sample are indicated in brackets. No values for the *fls-1* X Col4x is presented as the seed weight was very low (< 0.0000001 g). Significant differences were shown by a Mann-Whitney test: *, $P \leq 0.05$; **, $P \leq 0.01$.

3.2.2.2 Classification of mature seed morphology resulting from crosses between FBP mutant seed parents and Col 4x pollen parents

From the pilot study, morphological data was collected for mature seeds derived from crosses between Col4x pollen parents and all six of the FBP mutant lines utilised including the three background ecotypes.

Seeds derived from the *fls-1* X Col4x had an extreme abortive developmental phenotype compared to the control (Col2x X Col4x): seeds were irregular in shape and were significantly smaller (Figure 3.9.C).

All seeds derived from the *fls-1* X Col4x were considered to be non-viable being weak and collapsed. Seeds derived from crosses between *tt4-1*, *ttg2-1* and *tt10-5* and Col4x pollen parents were generally smaller than their control L*er*2x X Col4 (Figure 3.9.D, E, F and G).

The crosses between *tt12-1* and *tt16* and Col4x were also morphologically different from their control Ws2x X Col4x, with seeds derived from*tt16* being smaller and those derived from *tt12-1* being irregularly shaped (Figure 3.9.H, I and J).

In an attempt to categorise the seeds derived from each cross they were classified as a) plumb and burst seeds (both rescue seeds) and b) shrivelled seeds (abortive seed) according to their phenotype before statistical analysis (illustrated by arrows in Figure 3.9). Taking all the data on seed development together, it appears that shrivelled seed results from unregulated overgrowth followed by seed collapse and shrivelling during desiccation – this abortive phenotype results in small seeds and is linked to perturbation of endosperm and embryo development. Whereas burst seeds are those that result from regulated overgrowth during seed development and the large embryo ruptures the seed coat late in development. A plump seed is complete in form, well rounded and of a large size.

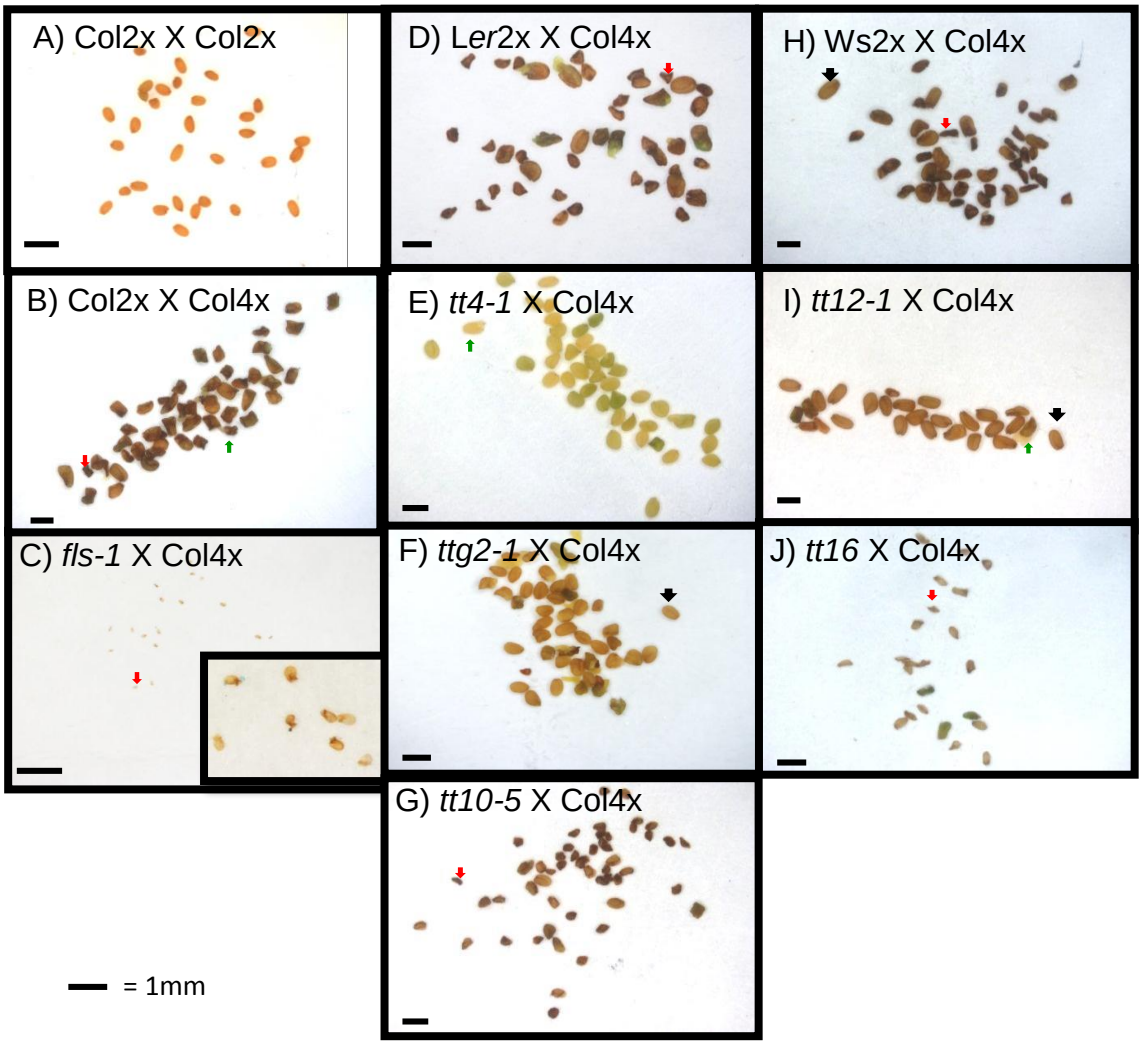


Figure 3.9: Analysis of morphology and size of seeds resulting from crosses between different FBP mutant seed parents (in varying ecotypes) and Col4x pollen parents. Two classes of seeds can be distinguished and then separated based on their viability: a) 'plump' and 'burst' seed phenotypes are generally viable and b) 'shrivelled' phenotypes are generally not viable. All the images of mature dry seeds were taken at the same light intensity and magnification, to facilitate morphological comparisons. Black arrows indicate plump seeds; burst seeds are indicated by green arrows and shrivelled seeds by a red arrow). Scale bar = 1mm.

The percentage of plump and burst seeds was then calculated for each silique and means were plotted with a standard error also indicated (Figure 3.10). As anticipated *fls-1* X Col4x had the lowest plump and burst percentage of all the seed groups derived from crosses between FBP mutants and Col4x pollen parents. The control Col2x X Col4x cross also resulted in very low numbers of plump/burst viable seeds

In the L*er* ecotype, the *tt10-5* X Col4x cross also had a low percentage of plump and burst seeds compared to *tt4-1*, *ttg2-1* and their control crossed to Col4x, indicating that most of these seeds were non-viable. Strikingly the L*er* 2x X 4x control including the *tt4-1* and

ttg2-1 L*er* lines rescued Col4x induced seed lethality compared to Col2x X Col4x crosses.

Regarding crosses using the Ws background as a seed parent, it is clear that seeds resulting from Ws ecotype crossed to Col4x, exhibit a smaller percentage of plump and burst seeds in comparison to the L*er* ecotype. Moreover, the plump and burst proportion in *tt16* X Col4x seeds was lower than *tt12-1* X Col4x and the control with these seeds appearing highly shrivelled and likely non-viable. Interestingly seeds resulting from the *tt12-1* X Col4x cross had a very high mean of plump and burst seeds indicating that this mutant has a significant impact on rescuing Col4x-induced lethality in this ecotype (Figure 3.10).

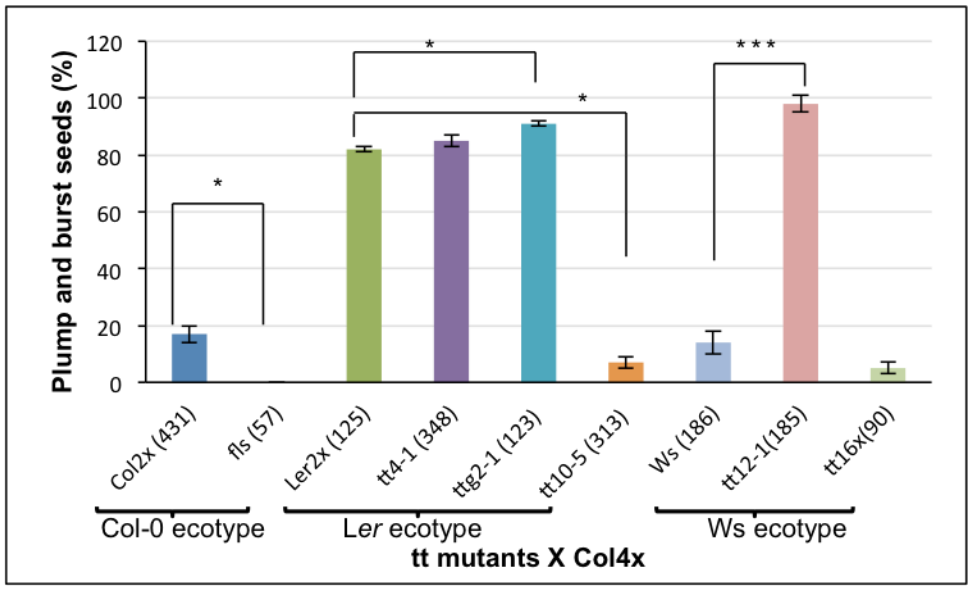


Figure 3.10: The average percentages of plump and burst seeds resulting from crosses between different FBP mutant seed parents (in varying ecotypes) and Col4x pollen parents. Plump and burst seeds were considered as viable. Error bars represent standard error. Sample sizes (**n**) are in brackets. Significant differences were shown by a Mann-Whitney test: *, $P \leq 0.05$; ***, $P \leq 0.005$.

3.2.3 Summary of a preliminary data

A summary of the data collected in this section is presented in Table 3.1. Rescue ratios are presented in Table 3.1[4].

[4] The ratio of a mean weight of mature seed and the percentage of plump and burst seeds were calculated as in section 2.2.4.

Table 3.1: Pilot study quantitative data resulting from crosses between different FBP mutant seed parents (in varying ecotypes) and Col4x pollen parents. Mature seed weight is one of the most reliable indicators of the degree of effectiveness for FBP mutant rescue of Col4x induced lethality. The order of data derived from crosses involving FBP mutants in this table thus runs from heaviest to lightest mean seed weight. Values are described as the mean ± S.E., with sample sizes (**n**) in brackets. Embryo stage data: black-shaded cells represent where observation of peripheral endosperm cellularisation was evident. **Abbreviations: *****: background ecotypes; ******: preliminary data *fls-1* mutant cross has very low weight to weigh (< 0.0000001 g) and low plump and burst percentage; **nd**: no data; ◆: no rescue ratio values obtained.

Genotype	**Seed development at 5, 7 and 9 DAP**												**Mature seeds**			
	Embryo stage			**Embryo sac area (μm ²)**			**Chalazal endosperm area (μm ²)**			**Number of nodules**			**Seed weight (μg)**	**%Plump & burst seed**	**Rescue ratio (weight)**	**Rescue ratio % (plump and burst)**
	5 DAP	**7 DAP**	**9 DAP**	**5 DAP**	**7 DAP**	**9 DAP**	**5 DAP**	**7 DAP**	**9 DAP**	**5 DAP**	**7 DAP**	**9 DAP**				
Col2xXCol 2x (reference cross)	heart	torpedo	curled cotyledon	nd	nd	nd	nd	nd	nd	0	0	0	17±3 (250)	nd	◆	◆
Col2x *	early heart	underdeveloped & abnormal	nd	6264 (9)	58738 (8)	nd	157(9)	11411 (8)	nd	1(9)	2(8)	nd	26±1 (431)	17 ± 3	◆	◆
Ws2x *	globular	nd	abnormal globular	nd	nd	nd	nd	nd	nd	nd	nd	nd	27±4 (186)	14 ± 4	◆	◆
Ler2x *	globular	heart	nd	32167 (9)	37872 (8)	nd	1822 (9)	3269 (8)	nd	1.11 (9)	0.78 (8)	nd	53 ± 0.8 (125)	82 ± 1	◆	◆
***ttg2-1* (L*er*)**	heart	early torpedo	nd	28902 (6)	52709 (9)	nd	2324 (6)	11006 (9)	nd	0.22 (6)	1 (9)	nd	56±0.2 (123)	91 ± 0.9	1	1.1
***tt12-1* (Ws)**	globular	nd	walking stick	25635 (7)	nd	nd	1048 (7)	nd	nd	0.11 (7)	nd	nd	47±3 (185)	98 ± 3	2	6.4
***tt4-1* (L*er*)**	heart	late heart	nd	18484 (6)	33318 (7)	nd	1100 (6)	1903 (7)	nd	0.67 (6)	1.22 (7)	nd	43±4 (348)	85 ± 2	0.8	1
***tt10-5* (L*er*)**	globular	abnormal globular	nd	30946 (9)	32345 (9)	nd	1385 (9)	1913 (9)	nd	0.78 (9)	0.67 (9)	nd	27±3.2 (313)	7 ± 3	0.5	0.09
***tt16* (Ws)**	overgrown globular	nd	arrested globular	19271 (8)	nd	56308 (5)	659 (8)	nd	7055 (5)	1.33 (8)	nd	2.4 (5)	8±0.3 (90)	5 ± 2	0.3	0.4
fls-1* (Col-0)*	globular	globular	nd	30738 (2)	53967 (2)	44746 (5)	785 (2)	1731 (2)	1003 (5)	0.44 (2)	0.44 (2)	2.2 (5)	0 (57)	0	0	0

In conclusion, the preliminary data presented in the previous sections and summarised in Table 3.1 has shown that both mutations in the FBP and different *A. thaliana* ecotypes influence seed development and mature seed phenotypes in crosses to Col4x pollen parents. In order to understand the effect of different mutations in the FBP on seed development in such interploidy crosses, it was decided that a further study should be initiated utilising a single Arabidopsis ecotype (Col-0) as the seed parent carrying a wide range of FBP T-DNA insertion mutants. This would eliminate the inter-ecotype variation described in this section of the thesis and also avoid the effects of potential unknown random mutations that are usually associated with ethyl methanesulfonate (EMS) mutants[5]. The next section describes the establishment of the necessary lines and the results of the study.

3.2.4 Isolation of homozygous FBP mutant lines in the Col-0 ecotype

Genes for all major enzymatic steps of the FBP pathway (*TT4*, *TT5*, *TT6*, *TT7*, *TT3*, *FLS*, *TT18/11*, *BAN*, *ANL1*, *TT19/14*, *TT12*, *TT10*, and *AHA10*) were targeted in this approach including a regulatory factor (*TTG2*). For each FBP gene, two to three independent insertion lines alleles were ordered from the NASC stock centre. Lines were genotyped according to section 2.1.1.

Once homozygous lines were isolated RT-PCR was utilised to determine if the T-DNA insertions resulted in gene knockouts or knockdowns (Fig 3.11). Assessment of gene function in these lines was not based on RT-PCR alone and also incorporated information on seed coat colour, the location of the T-DNA in the relevant gene and previously published works. The colour of FBP mutant seed coats vary from yellow to dark brown (Table 3.2); for example, *tt4, tt6,* were yellow; *tt5* was pale tan brown; *tt7* was pale brown; *tt3, tt18/11* were dark yellow; *fls* was brown; *ban* and *anl1* were dark brown; *tt19/14* was tan brown; *tt12, aha10, tt10* and *ttg2* were very pale brown. The T-DNA insertion lines in the *TT6, TT11/18, TT12, TT7* and *ANL1* genes were in open reading frames, whereas, in *TT5, TT3, FLS, BAN, TT10* and *TTG2* the insertions were in promoter regions, and *TT4, TT14/19* and *AHA10* had insertions within introns. The section below summarises

[5] All mutants studied so far were EMS mutants.

the information for each line utilised in this study and draws from data presented in Fig 3.11 and Table 3.2:-

TT4 - The Gabi-KAT line (GK_304D03.02) is defined as having\ a T-DNA insert in intron region of *At5G13930* (TT4). RT-PCR results showed a decrease in the expression of *TT4* in this mutant line. This knockdown mutation was enough to cause a yellow seed coat and in all likelihood the remaining transcript would produce an aberrant non-functional protein. Thus, this line was considered a probable functional knockout.

TT5 - The insertion line of SALK_034145.51.00.x had a T-DNA insertion in the 5' untranslated region (UTR) of *At3G55120* (TT5). A transcript was detected by RT-PCR and thus based on this data cannot definitively be considered a *TT5* knockout. However, Bowerman *et al.* (2012) reporting on *tt5–3* has a similar SALK accession code (SALK_034145 vs. SALK_034145.51.00.x in our case), where the T- DNA insertion was reportedly in the coding sequence. The seed phenotype (pale tan brown) does suggest a knockout or knockdown of the gene in the line used in this study.

TT6 – SALK_068963.53.50.x has its T-DNA located in the second exon of *At3G51240* (TT6). No transcript was detected by RT-PCR. Furthermore, the *tt6* mutant seed coat phenotype was yellow – Taken together this indicates that this line is a true knockout.

TT7 – The SAIL_573_G12 insertion line had the T-DNA located in exon 3 of *At5G07990* (TT7). RT-PCR suggested a knockdown as some transcript was detected. The seed coat phenotype was pale brown indicating a phenotypic effect of the insertion in *At5G07990* meaning that the phenotype was affected. Given the exonic location of the insertion, reduced transcript levels and a marked though not extreme seed coat phenotype this line can be cautiously assigned knockout status.

TT3 - The SALK_099848.56.00.x line T-DNA is inserted in the 5' UTR region of *At5G42800* (TT3). Residual transcript level was detected by RT-PCR however, the dark yellow seed phenotype of *tt3* does suggest a strong knockdown of TT3 in this line.

FLS - The SALK_009992.49.80.x T-DNA is inserted in 3' UTR region of *At5G08640* (FLS). RT-PCR data demonstrated this line to be a knockdown. Seed coat colour was similar to WT colour as would be expected for a lesion in this side branch of the pathway.

TT11/18 - SALK_073183 has the T-DNA located in exon 2 of *At4G22880* (TT11/18). No transcript level was detected by RT-PCR utilising primers around the region of the T-DNA insertion area. Importantly the *tt11/18* mutant seed coat phenotype was dark yellow indicating this to be a knockout line. Moreover, *tt11/18* knockout status has previously

been confirmed by others - Abrahams *et al.* (2003), Bowerman *et al.* (2012) and Appelhagen *et al.* (2014).

BAN - SALK_122474.20.75.x has a T-DNA located in the 5' UTR region of *At1G61720* (BAN). RT-PCR detected a transcript. The mature seed coat phenotype indicated darker colouration than that of WT and immature seeds displayed a reddish colouration indicating precocious accumulation of anthocyanin typical of BAN mutants. Moreover, mass spectrometry data indicated that this *ban* mutant accumulated higher levels of soluble procyanidin (Figure 5.5). Taken together this data suggests a substantial knockdown of BAN despite transcript being detected by RT-PCR (saturating PCR was carried out which would not reflect true transcript levels).

ANL1 - SALK_049338.56.00.x has a T-DNA insertion in exon 2 of *At5G17050* (ANL1). RT-PCR results indicated that this line was a true Knockout as no transcript could be detected. Seed coat colour was darker than that of WT.

TT14/19 - SALK_105779 line has a T-DNA inserted in intron 2 of *At5G17220* (TT14/19). RT-PCR results found no transcript for this gene. Moreover, *tt14/19* (SALK_105779) had a tan brown seed coat, which is a characteristic of several FBP mutants. The RT-PCR results were consistent with the findings of Wangwattana *et al.* (2008) and Appelhagen *et al.* (2014). Moreover, Kubo *et al.* (2007) reported lower levels of anthocyanin in plant tissues, which reflects the importance of TT14/19 in anthocyanin transport.

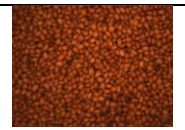
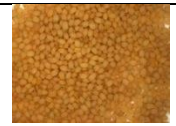
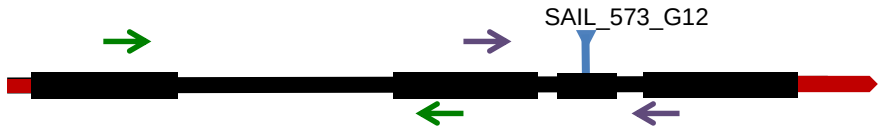
TT12 - GK_797D03.04 has a T-DNA insertion in exon 2 of *At3G59030* (TT12). No transcript was detected by RT-PCR, and the seed coat had very pale brown colour. The RT-PCR results were consistent with results by Kitamura *et al.* (2010) and Appelhagen *et al.* (2014) and thus, a knockout was confirmed.

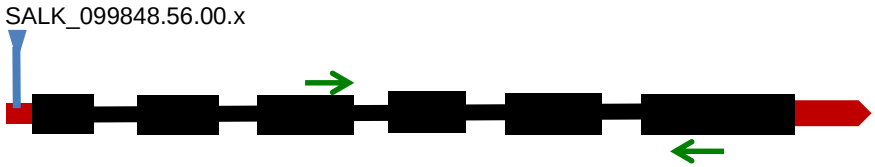
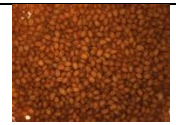
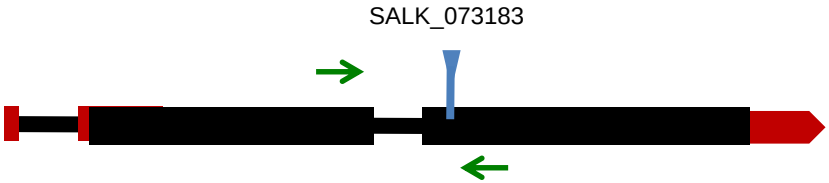
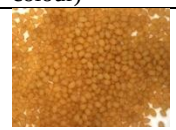
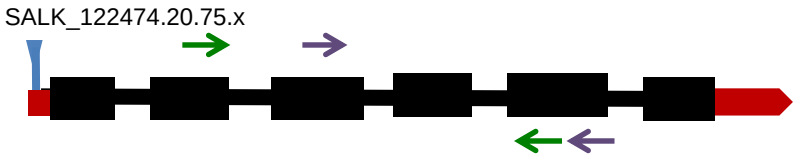
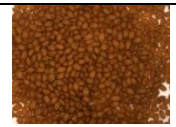
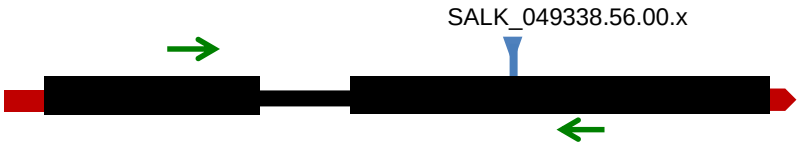
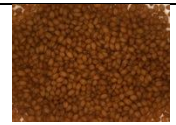
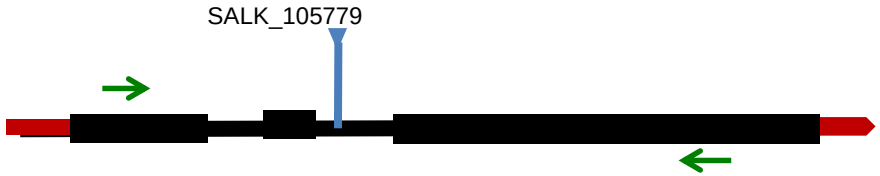
TT10 - GK_146E10.02 has a T-DNA insertion in the 5' UTR region of *At5G48100* (TT10). No transcript was detected by RT-PCR and seeds had a very pale brown phenotype. The RT-PCR results were consistent with results by Ishihara (2007) and Appelhagen *et al.* (2014) and thus, this line was confirmed as a knockout.

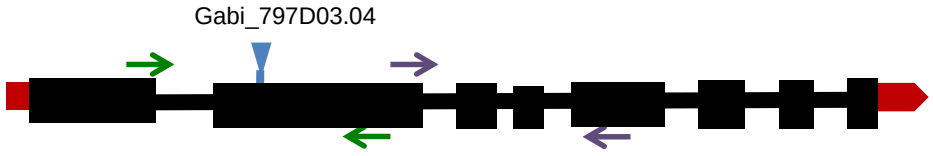
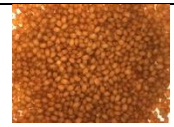
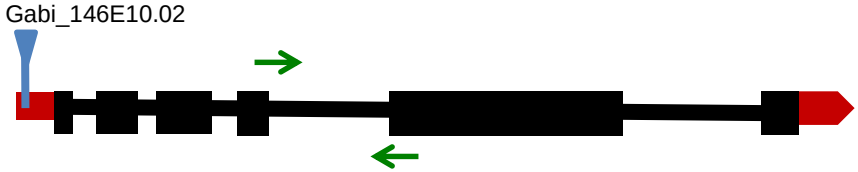
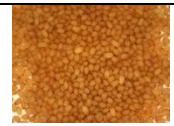
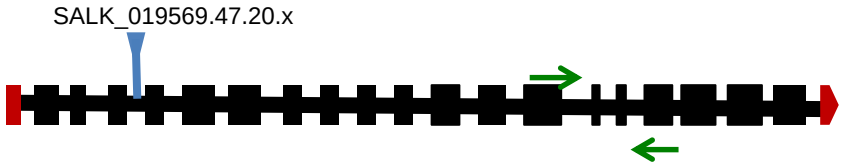
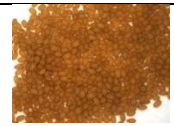
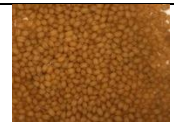
AHA10 - SALK_019569.47.20.x has a T-DNA insertion in intron 4 of *At1g17260* (AHA10). A very low level of the transcript was detected by RT-PCR and the seed coat colour of this mutant line was very pale brown. The RT-PCR results were consistent with those of Baxter *et al.* (2005) and this line was considered to be a functional knockout of AHA10.

TTG2 - SALK_148838.51.70 had a T-DNA insertion in exon 1 of *AT2G37260* (TTG2) and the seed coat was of a very pale brown colour. Together these indicate a knockout of this gene. RT-PCR provided inconclusive data regarding the transcript for TTG2 from this line however given the pale seed phenotype and position of the T-DNA insertion this line was considered to carry a functional knockout of TTG2.

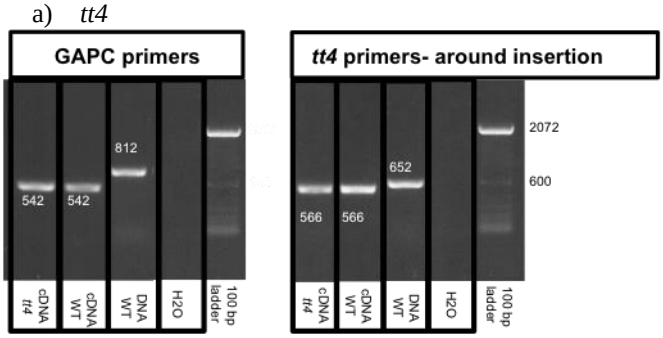
Table 3.2: Characterisation of FBP T-DNA insertion lines based on insertion site, non-quantitative RT-PCR and seed phenotype. Abbreviations: 5'UTR: ; exon: ; intron: ; 3'UTR: ; First pairs of primers: ; Second pairs of primers: . The results of the RT-PCR are presented in figure 3.11.

Flavonoid biosynthesis genes and regulatory gene	**Gene structure in Col-0 ecotype**	**RT-PCR results (transcript present? Yes or No)**	**Seeds coat colour of FBP mutants in Col-0 ecotype**
WT (Col-0)			 Brown
TT4 (At5G13930)		Yes	 Yellow
TT5 (At3G55120)		Yes	 Pale tan brown
TT6 (At3G51240)		No	 Yellow
TT7 (At5G07990)		Yes	 Pale brown

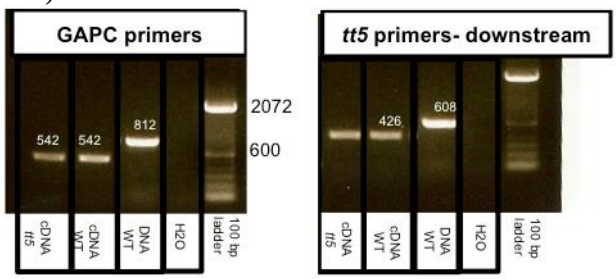
TT3 (At5G42800)		Yes	 Dark yellow
FLS1 (At5G08640)		Yes	 Brown (similar to WT colour)
TT11/TT18/ TDS4-4 (At4G22880)		No	 Dark yellow
BAN (At1G61720)		Yes	 Dark brown (darker than WT colour)
ANL1 (At5G17050)		No	 Dark brown(darker than WT colour)
TT14/TT19 (At5G17220)		No	 Tan brown

TT12 (At3G59030)		No	 Very pale brown
TT10 (At5G48100)		No	 Very Pale brown
AHA10 (At1g17260)		Yes	 Very pale brown
TTG2 (At2G37260)		Yes	 Very pale brown

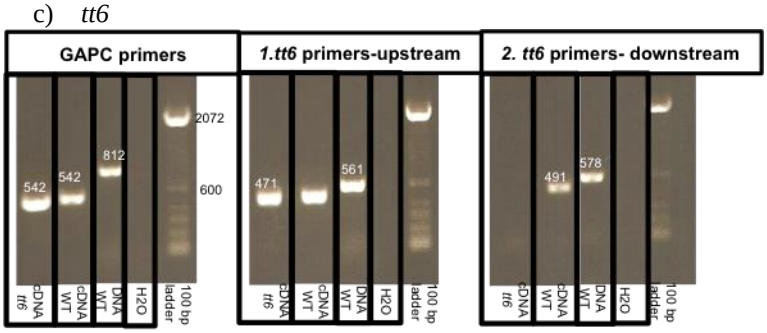
a) *tt4*



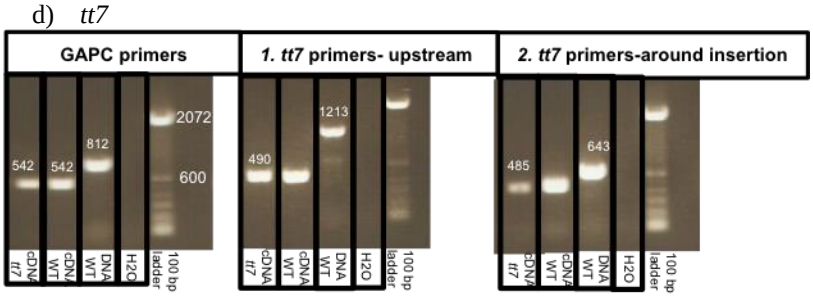
b) *tt5*



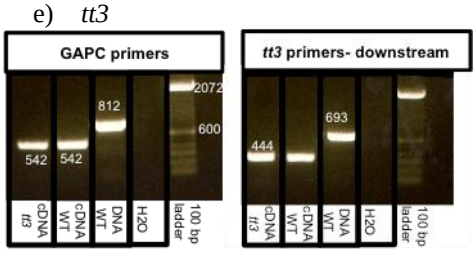
c) *tt6*



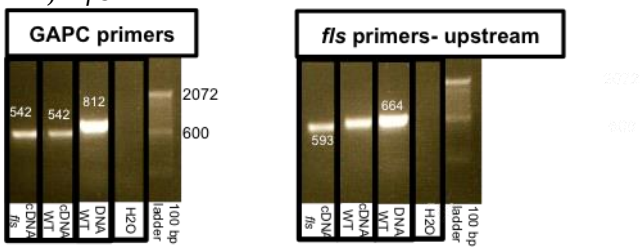
d) *tt7*



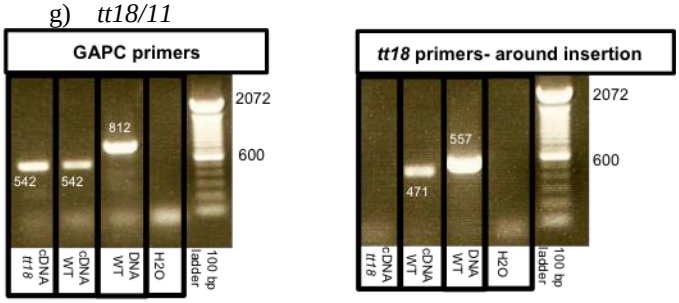
e) *tt3*



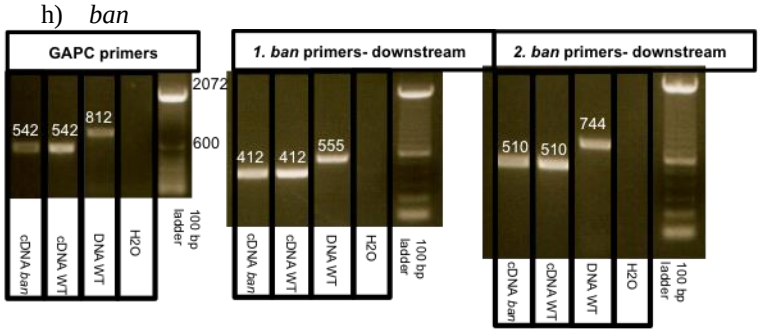
f) *fls*



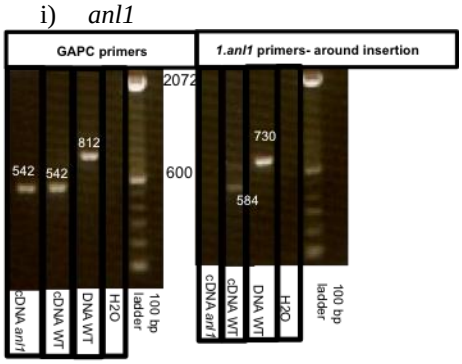
g) *tt18/11*



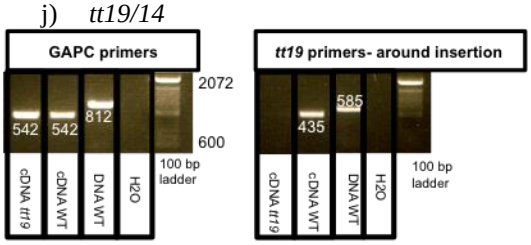
h) *ban*



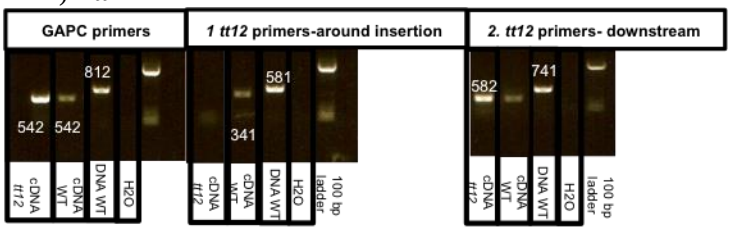
i) *anl1*



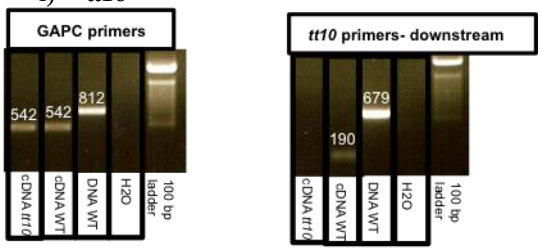
j) *tt19/14*



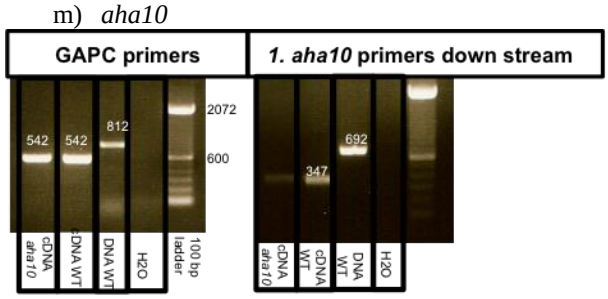
k) *tt12*



l) *tt10*



m) *aha10*



n) *ttg2*

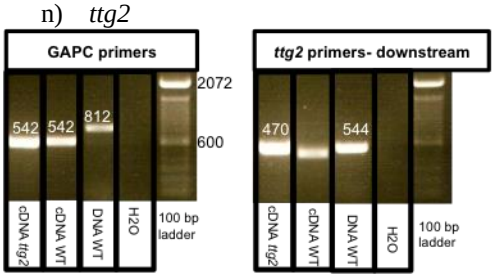


Figure 3.11: RT-PCR of cDNA FBP mutants and WT from 7 DAP siliques in Col-0 ecotype. GAPC was used as a loading control and transcripts were detected from mutant cDNA, WT cDNA, WT genomic DNA but not with the water as negative control. The DNA ladder was 100 base pair (bp). Details of upstream and downstream specific primers for *tt* mutants are shown in Table 3.2. PCR product size is written above the band on each figure. The primers and PCR conditions are detailed in Table 2.5.

3.2.5 Developmental analysis of seeds by confocal microscopy following crosses between maternal FBP mutants in Col-0 ecotype and Col4x pollen parents

In the following sections, the same seed characteristics will be described as those covered in the previous section. Here FBP T-DNA insertion lines are utilised that are all carried in the *Arabidopsis* Col-0 background in contrast to the pilot study described in section 3.2.1 where EMS/point mutation lines were used in crosses to Col4x pollen parents.

3.2.5.1 What effect do FBP mutations in the Col-0 background have on Col4x lethality?

Confocal microscopy analysis revealed well-defined differences in progression of seed development between FBP mutants crossed with Col4x and the control cross (Col2x X Col4x). Figures 3.12.A and B illustrate FBP mutant seed phenotypes at 5 and 7DAP following interploidy crosses to Col4x pollen parents. Embryo growth was inhibited and remained at an early heart stage between 5 and 7DAP in the cross involving *ttg2,* and at the heart stage in crosses involving *tt6, tt3* and *ban.* However, embryos developed from the globular stage at 5 DAP to heart stage at 7DAP in crosses involving *tt11/18* and *aha10*. Interestingly, in the crosses involving the rest of the FBP mutants at 5DAP, the embryo reached a) a globular stage (in *anl1*, *tt14/19* and *tt10*), b) an early heart stage in Col2x, *tt4* and *tt7* and, c) the heart stage in *tt5*, *fls* and *tt12*.

Moreover, there was a significant delay in development by 7DAP, forming an abnormally overgrown, globular-like embryo structure in crosses involving Col2x, *tt4*, *tt5*, *fls*, *anl1*, *tt14/19*, *tt10* and *tt12*. In contrast, *tt7* was an underdeveloped and abnormal embryo structure. Furthermore, it was clearly observed that in *tt7* X Col4x, *ban* X Col4x and *anl1* X Col4x crosses, the central peripheral endosperm showed signs of over-proliferation and delay in endosperm cellularisation at 5 DAP.

The chalazal endosperm size in all crosses increased and some of them were even vacuolated between 5DAP and 7DAP (Col2x X Col4x, *tt4* X Col4x, *tt5* X Col4x, *tt6* X Col4x, *fls* X Col4x, *anl1* X Col4x, *ban* X Col4x). In many FBP mutant crosses collapse of chalazal endosperm was evident along with nodule formation on the peripheral endosperm (e.g. *ban* X Col4x and *tt10* X Col4x at 5 DAP and *tt11/18* X Col4x, *ban* X Col4x, *tt12* X Col4x, *tt10* X Col4x at 7DAP). The micropylar peripheral endosperm (which forms a

denser tissue around the embryo base and the suspensor) was unusually vigorous and started to engulf the embryo in all *tt* mutant crosses.

Seed size appeared to increase with seed development in most crosses from 5 to 7DAP, with the exception of seeds resulting from *tt10* X Col4x crosses, which remained similar in size between 5 and 7DAP.

Finally, seeds descending from *ban* X Col4x and *anl1* X Col4x at 9DAP had lethal shrivelled phenotype (Figures 3.13).

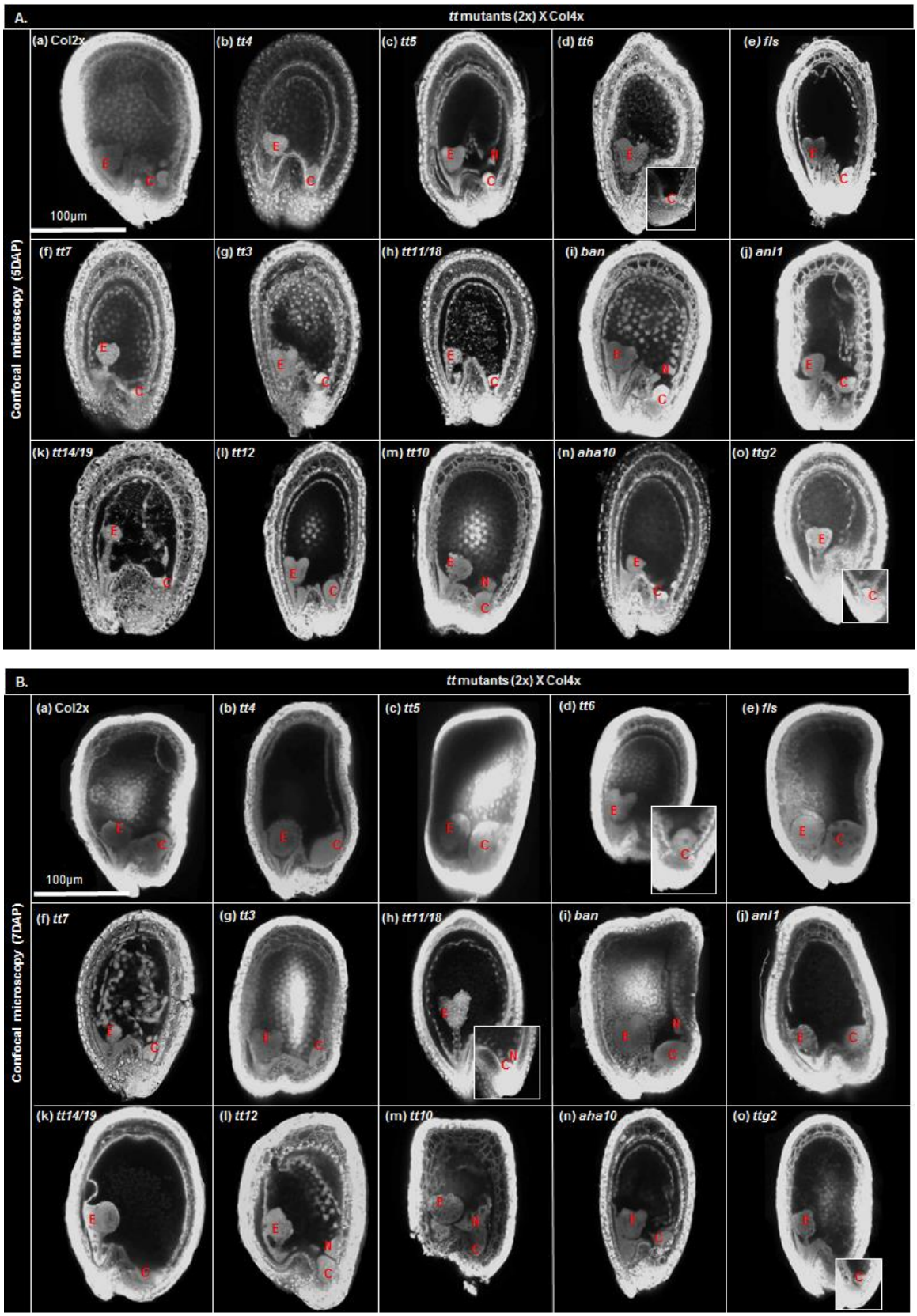


Figure 3.12: Effect of maternal FBP mutations on seed development in crosses with Col4x pollen parents. A. Confocal laser-scanning photomicrographs of *tt* mutants in Col-0 ecotype in interploidy crosses at 5DAP. **B.** Confocal laser-scanning photomicrographs of *tt* mutants in Col-0 ecotype crossed to Col4x at 7DAP. (**a**) Control cross, Col2x X Col4x, (**b**) to (**o**) *tt* mutants in Col-0 ecotype crossed to Col4x. **E**, embryo; **C**, chalazal endosperm; **N**, nodules. All images were taken at 20x magnification. The embryo in *tt7* X Col4x

cross at 7DAP (**f**) was very small and arrested at globular stage. Scale bar = 100µm in all images.

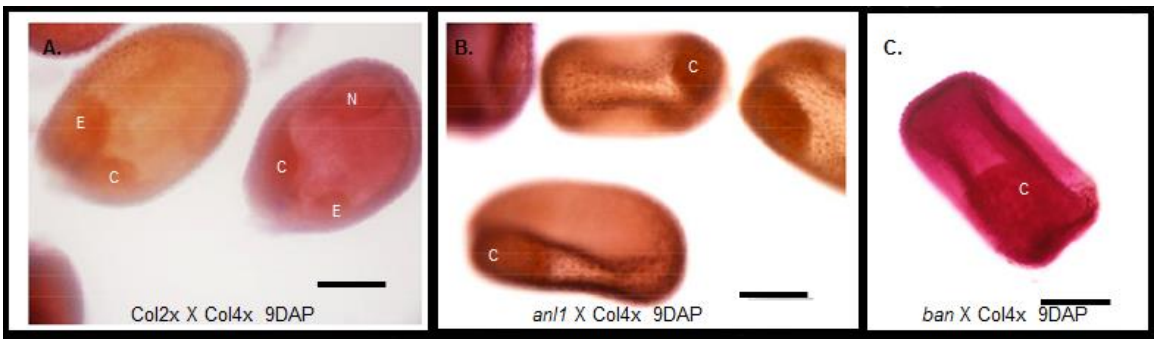


Figure 3.13: Seed morphology at 9 DAP resulting from *anl1*XCol4x and *ban* XCol4x. (**A**) Light microscopy images display the seeds in the control cross Col2x X Col4x, (**B**) *anl1* X Col4x and (**C**) *ban* X Col4x. *anl1* X Col4x and *ban* X Col4x seeds collapsed and had loss of structures compared to the control cross. Photos (**A**) and (**B**) were taken by Matthew Udakis. **E**, embryo; **C**, chalazal endosperm; **N**, nodules. Seed image captured at 10x magnification. Scale bar =200µm.

3.2.5.2 Increases in embryo sac area during seed development is differentially affected by maternally carried Col-0 FBP mutations in crosses to Col4x pollen parents

As can be seen, in figure 3.13 many of the crosses between FBP mutants in the Col-0 background and Col4x pollen parents resulted in dramatic increases in the embryo sac area between 5 and 7 DAP (*tt4, tt5, tt6, tt3, ban, anl1, tt19/14, tt10, aha10* and *ttg2*). This contrasts with the control Col2x X Col4x cross where expansion of the embryo sac between 5 and 7DAP was small. Several mutants mirrored the data of the control (*fls, tt7, tt18/11* and *tt12*) also having much reduced or arrested expansion of the embryo sac over this period of seed development. *tt19/14* X Col4x resulted in the smallest embryo sac area at 5DAP, with a mean of 23655 µm^2, whereas, *tt18/11* X Col4x had the largest embryo sac area at 5DAP, with a mean of 61442 µm^2. By 7DAP, seeds from the *tt4* X Col4x cross had the greatest embryo sac area with a mean of 130296 µm^2 whereas, *tt7* X Col4x seeds had the lowest embryo sac area, with a mean of 42854 µm^2.

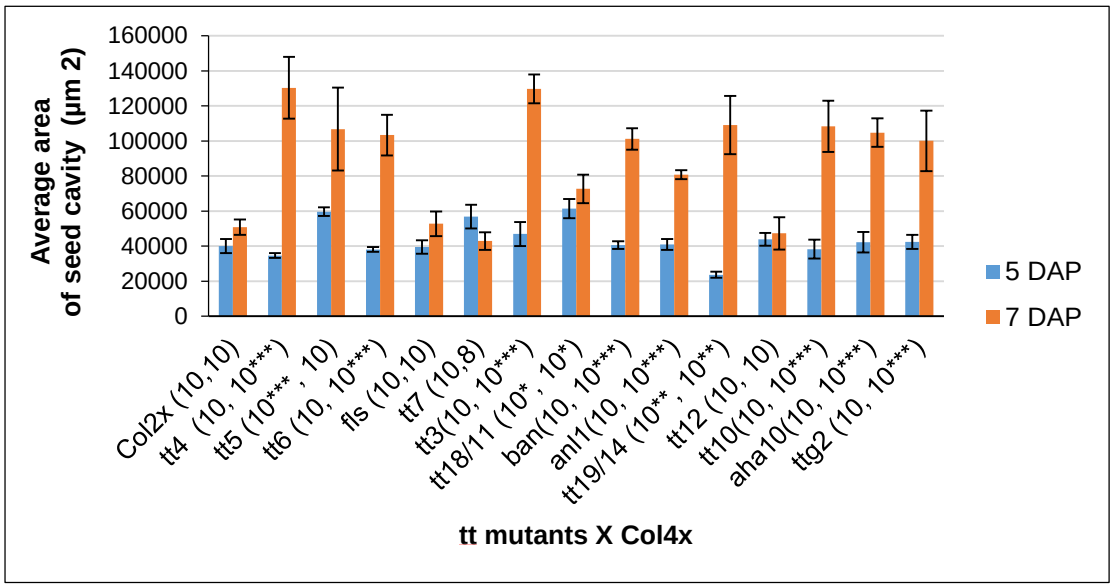


Figure 3.14: Mean measurements of embryo sac area at 5 and 7 DAP in seeds resulting from FBP mutants in Col-0 crossed with Col4x pollen parents. Embryo sac areas are represented by blue and orange bars to denote 5 and 7DAP respectively. Embryo sac area increased in all crosses between 5 and 7DAP except for *tt7*. Mean areas were measured in µm^2, being estimated from confocal micrograph images captured at 20x magnification. Error bars represent standard error. Sample sizes (**n, n**) are in brackets; sample size is 10 seeds in all crosses at 5 and 7 DAP except in *tt7* X Col4x is 8 seeds at 7DAP. Seeds were collected from two siliques. Significant differences were shown by a Mann-Whitney test: *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.005.

3.2.5.3 Increases in chalazal endosperm area during seed development is differentially affected by maternally carried Col-0 FBP mutations in crosses to Col4x pollen parents

Chalazal endosperm area was observed to increase during seed development from 5 to 7 DAP in all crosses, except for *tt7* (Figure 3.15). In many instances, the increase in area was dramatic in comparison to the Col2x X Col4x control (e.g. *tt4, tt5, tt3* and *anl1*). At 5DAP, the chalazal endosperm area in *fls* X Col4x cross was the largest, with a mean of 5384 µm^2, whereas seeds were resulting from the cross involving *anl1* with Col4x, it was the smallest with mean of 764 µm^2. At 7DAP, the chalazal endosperm area in *anl1* X Col4x cross was the largest, with a mean of 52555 µm^2, whereas in *tt7* X Col4x cross it was the smallest with mean of 1259 µm^2. Taken together the data indicate that maternally carried FBP mutations have a dramatic effect on chalazal endosperm development.

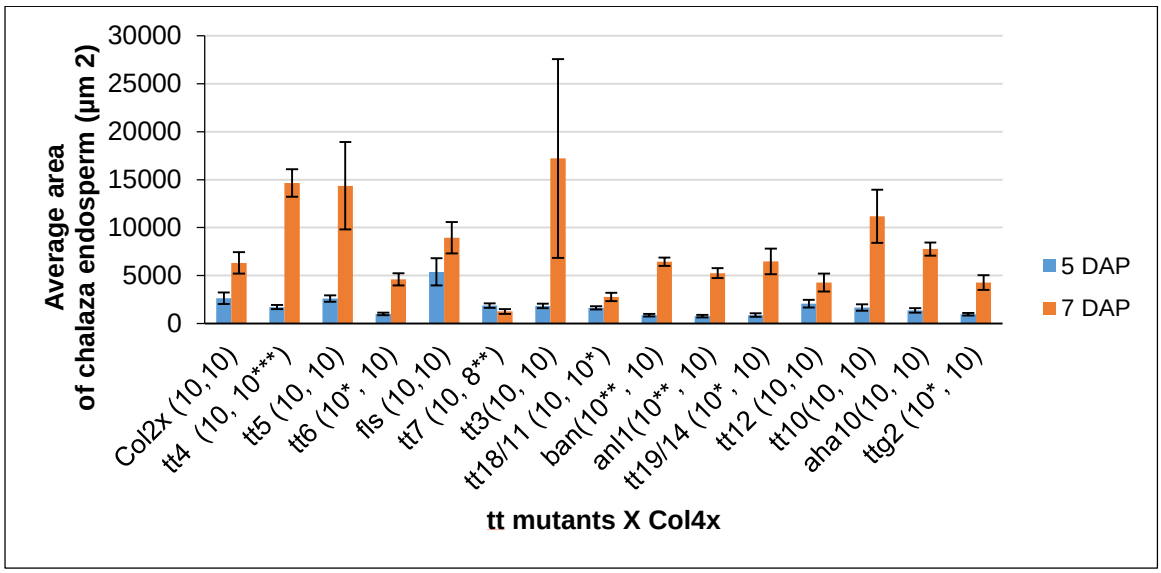


Figure 3.15: Chalazal endosperm areas at 5 and 7 DAP in seeds derived from crosses between maternally carried FBP mutations in the Col-0 ecotype crossed with Col4x pollen parents. Chalazal endosperm areas are shown for 5 DAP (blue bars) and 7DAP (orange bars). Chalazal endosperm area is seen to increase in all crosses except for that involving *tt7*. Areas were measured in μm^2 using confocal images captured at 20x magnification. Error bars represent standard error. Sample sizes (**n,n**) are in brackets. Seeds were collected from two siliques. Significant differences were shown by a Mann-Whitney test: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$.

3.2.5.4 Endosperm nodule number is differentially affected by maternally carried Col-0 FBP mutations in crosses to Col4x pollen parents

At 5 and 7DAP, the average numbers of nodules were counted in seeds generated from crosses between FBP mutants in the Col-0 ecotype and Col4x pollen parents (Figure 3.16). The number of nodules was the highest in *ban* X Col4x at 5 and 7DAP, with a mean of 1.9 and 2.4, respectively, whereas, in *tt19/14* X Col4x the number of nodules was lowest at 5 DAP, with a mean of 0.3. Moreover, the number of nodules in *tt10* X Col4x was smallest at 7 DAP, with a mean of 0.1.

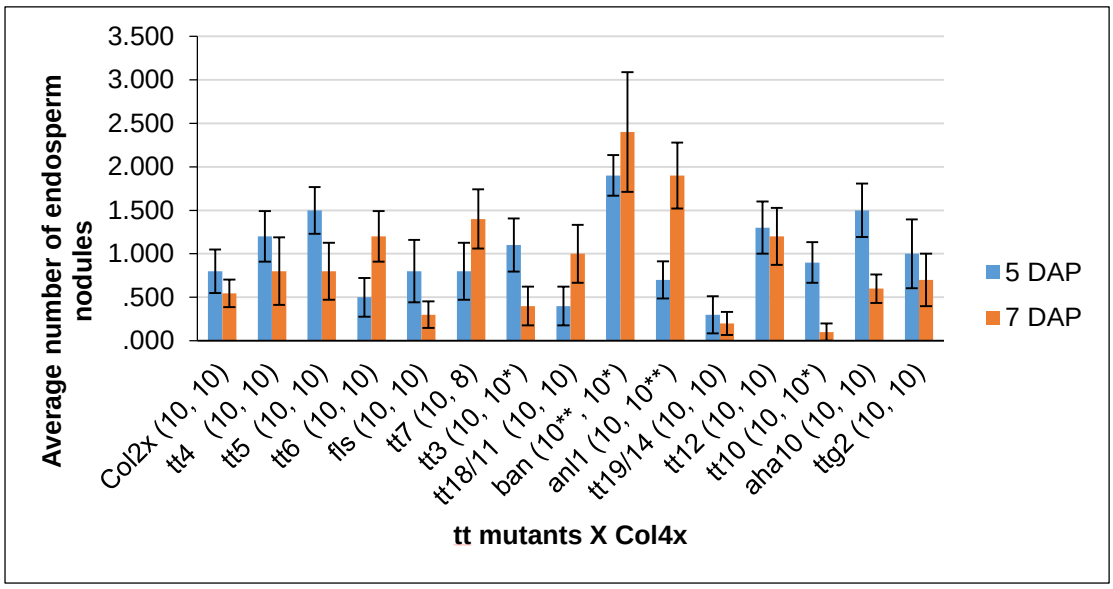


Figure 3.16: Average number of endosperm nodules in seeds derived from crosses between maternally carried FBP mutants in Col-0 ecotype and Col4x pollen parents. Blue and orange bars represent average nodule number at 5 and 7DAP, respectively. The mean number of nodules were accounted and estimated from confocal micrograph images captured at 20x magnification. Error bars represent standard error. Sample sizes (**n,n**) are in brackets. Seeds were collected from two siliques. Significant differences were shown by a Mann-Whitney test: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$.

3.2.6 Analysis of mature seed weight, phenotype and viability for seeds derived from crosses between FBP mutants in Col-0 ecotype and Col4x pollen parents

3.2.6.1 Mature seed weight is influenced by maternally carried Col-0 FBP mutations in crosses to Col4x pollen parents

Results showed a variation in seed weight amongst seeds derived from the various FBP mutant crosses (Figure 3.17). Mature seeds arising from *tt4* and *aha10* crossed with Col4x were the heaviest, having means of 25 µg and 24 µg respectively. Seeds derived from crosses involving *tt6*, *tt12* and *ttg2* also produced heavy seeds in comparison to seeds derived from the Col2x X Col4x control with means of 22 µg, 21 µg and 20 µg, respectively. On the other hand, several mutants (*anl1*, *tt7*, *tt10* and *tt5*) generated seeds that were lighter than the control cross with *anl1* producing the lowest weight seeds (6µg).

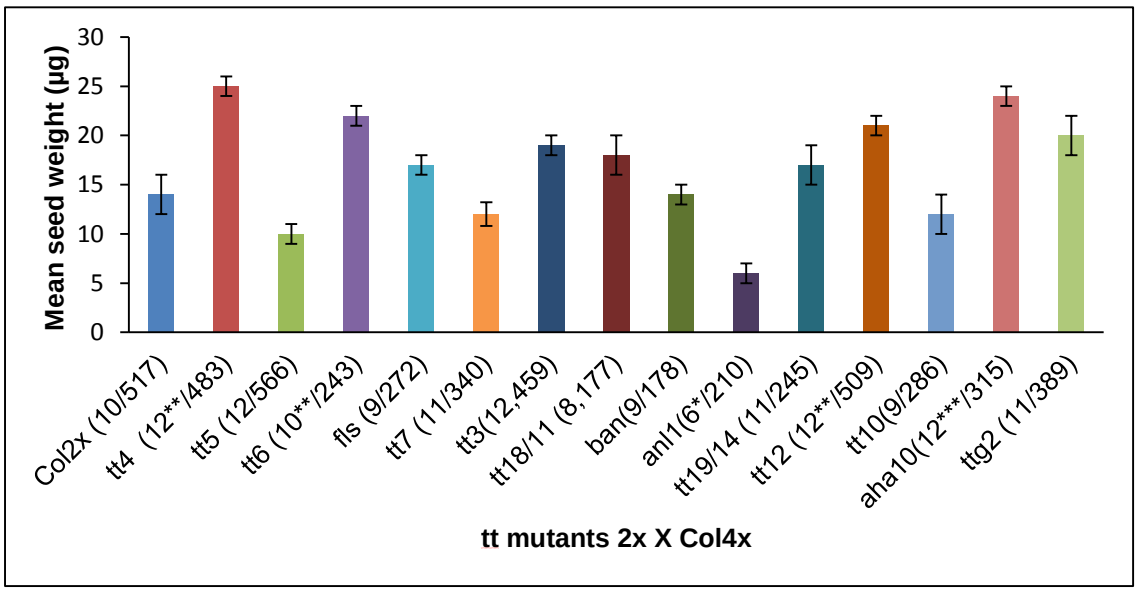


Figure 3.17: Mean weights of mature seeds derived from crosses between FBP mutants in the Col-0 ecotype and Col4x pollen parents. The weight of the seeds was measured in µg. Col2x X Col4x is the control cross. Error bars represent standard error. Numbers of siliques (**n**) and seed number per sample (**n**) are indicated in brackets (**n/n**). Significant differences were shown by a Mann-Whitney test: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$.

3.2.6.2 Mature seeds derived from crosses between maternal FBP mutants and Col4x pollen parents vary in phenotype

Most seeds resulting from crosses between the different FBP mutants and Col4x had an irregular shape; moreover, seeds could be placed into two distinct groupings based on their overall phenotype - plump and burst seeds (having a large seed size) and shrivelled seeds (having a small seed size). Both phenotypic classes were found in varying proportions in the seeds resulting from each cross. Mature seeds derived from crosses to Col4x pollen parents involving *ban, anl1*, *tt19/14* and *tt10* generally had smaller and irregularly shaped seeds (almost all seeds derived from these *tt* mutants had shrivelled seeds) (Figure 3.18.I-K and M) compared to the control cross. Moreover, crosses involving *aha10* and *ttg2* produced seeds that were larger than those derived from the control cross, and almost all had irregular plump and burst seeds (Figure 3.18.N and O). Interestingly, seeds descending from crosses between *tt4*, *tt6*, *tt18/11* and *tt12* with Col4x were noticeably more angular and larger than those from Col2x WT mothers, in addition to being plump and burst shaped (Figure 3.18.B, D, H and L). Seeds resulting from crosses involving *tt5*, *fls*, *tt7* and *tt3* were irregularly shaped with some being smaller in size, and shrivelled, whereas others were larger, plumper and burst than Col2x X Col4x seeds (Figure 3.18.C, E, F and G).

The resulting seeds from each cross were analysed by calculating the numbers of a) plumb and burst seeds (both considered 'rescue' seeds) and b) shrivelled seeds (lethal/nonviable seed) according to their phenotype (illustrated by arrows in Figure 3.19).

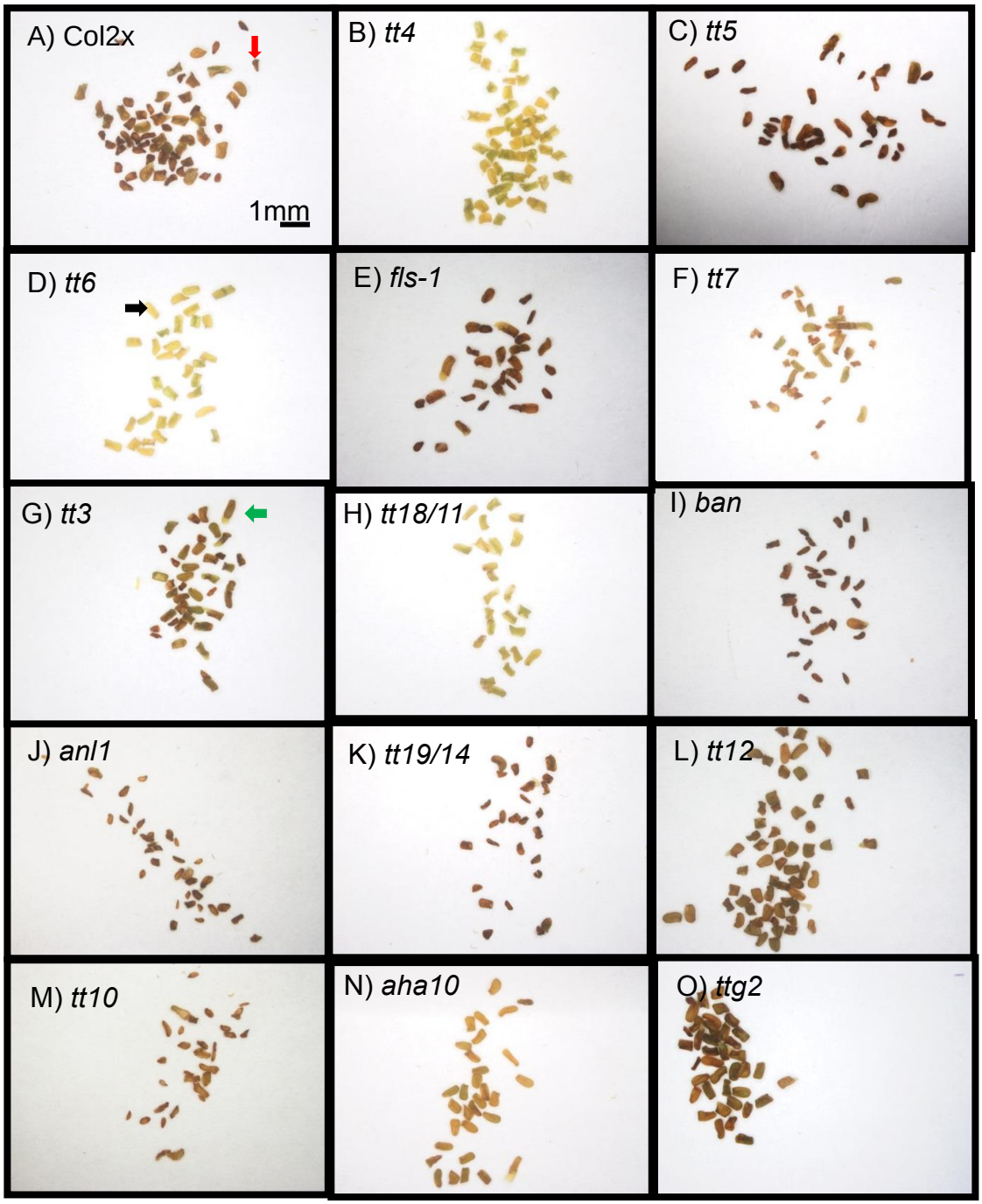


Figure 3.18: Morphology of seeds derived from crosses between FBP mutant maternal lines with Col4x pollen parents. Differentiating morphology and size of mature seeds resulted in classifying seeds into two classes based on their viability: a) 'plump' and 'burst' seed phenotypes fall into the rescue category, being considered viable b) 'shrivelled' seed phenotype falls into the lethal non-viable category. (**A**) Control cross Col2x X Col4x. (**B**) to (**O**) FBP mutant crosses with Col4x pollen. All the images of mature dry seeds were taken at the same light intensity and magnification, in order to make a comparison of seed shape and size. Seeds were classified and counted as plump (indicated using a black arrow), burst (indicated using a green arrow) and shrivelled (indicated using a red arrow). Scale bar = 1mm.

The percentage of plump and burst seeds were then calculated for each silique and a mean plump and burst percentage was plotted with a standard error also being indicated (Figure 3.19). Most FBP mutant lines produced higher percentages of plump and burst (rescue) seed than the Col2x X Col4x control (6%) with the exception of *anl1* and *tt10*. The highest proportion of plump and burst seeds resulting from crosses to Col4x pollen parents was recorded for *tt12* and *aha10* lines, with means of 54% and 45% respectively, followed by *tt3*, *ttg2* and *tt6*, with means of 34%, 28% and 27% respectively. In contrast, seeds resulting from crosses involving *anl1*, and *tt10* crossed to Col4x did not show any significant change in comparison to control cross (Figure 3.19).

The percentages of plump and burst seeds resulting from crosses with *tt4*, *tt7*, *tt19/14*, *tt5*, *fls*, *ban* and *tt18/11* were higher than the control cross, with an average percentage of 21%, 21%, 20%, 19%, 19%, 18% and 16%, respectively.

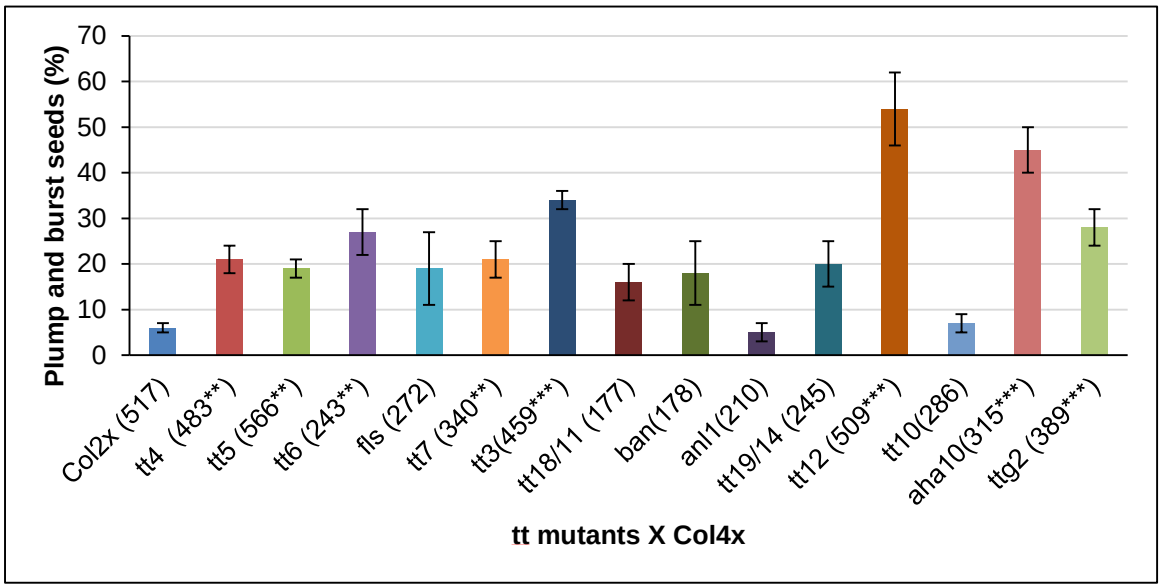


Figure 3.19: Mean percentage of plump and burst (rescue) seed phenotypes for seeds resulting from crosses between FBP mutant lines and Col4x pollen parents. Plump and burst mature seeds were counted as viable. Error bars represent standard error. Sample sizes (**n**) are in brackets. Seeds were collected from ten siliques. Significant differences were shown by a Mann-Whitney test: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$.

3.2.6.3 Viability of seeds derived from crosses between Col-0 FBP mutants and Col 4x pollen parents

A germination test was conducted to get a broader view of the effects of maternal FBP mutations and paternal excess on the seed phenotype. Germination of mature seeds derived

from crosses between different FBP mutants with Col4x was tested as described in section 2.2.6.

It should be noted here that the seeds that were tested by germination test were not, all, freshly collected stock; e.g. some of them were stored for months. However, variation in seed viability was evident, for example; *anl1*, *tt10* and *ban* had the lowest numbers of viable seeds, with mean germination values of 3%, 3% and 4%, respectively (lower than the control cross Col2x X Col4x– 7%), whereas *tt6* and *ttg2* had the highest germination, with means of 26% and 31% respectively. Moreover, crosses involving *tt3* and *aha10* mutants with Col4x resulted in seeds with relatively high germination frequencies: 17% and 19%, respectively. The levels of germination in crosses involving *tt5*, *tt7*, *tt4*, *fls*, *tt12*, *tt18/11*, *tt19/14* and *ban* were 8%, 8%, 7%, 7%, 7%, 6%, 5% and 4%, respectively (Figure 3.20).

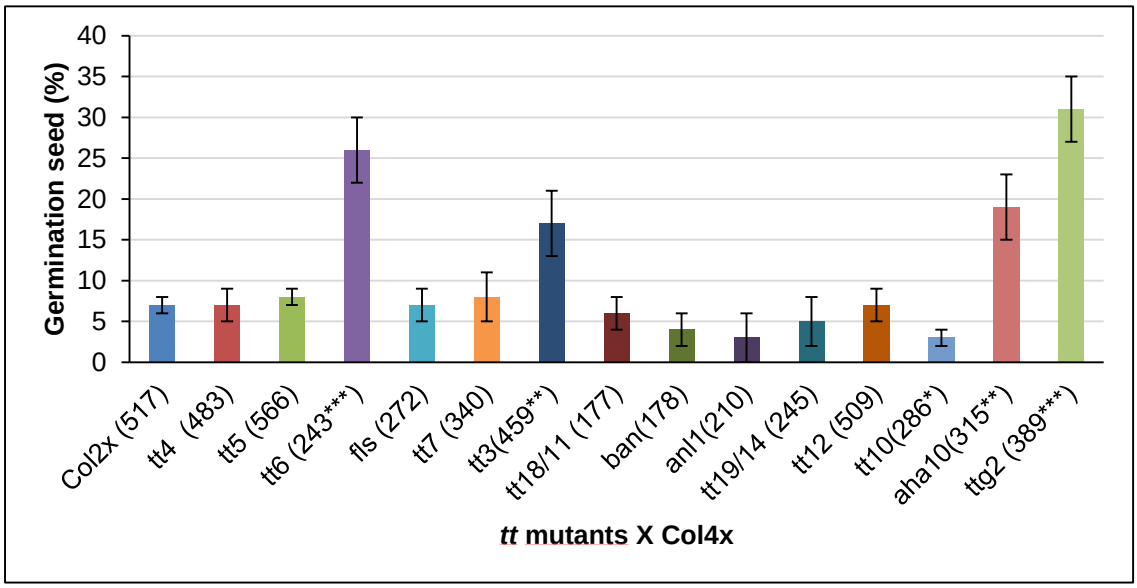


Figure 3.20: Mature seed viability (germination %) of seeds derived from crosses between Col2x FBP mutants and Col4x pollen parents. Most seeds resulting from crosses between FBP mutants in Col-0 ecotype with Col4x pollen parents had similarly low levels of viability to the Col2x X Col4x control cross. Error bars represent standard error. Sample sizes (**n**) are in brackets. Seeds were collected from ten siliques. Significant differences were shown by a Mann-Whitney test: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$.

3.2.7 Summary of FBP T-DNA insertion mutants data

All the data presented in the preceding sections relating to the progression of embryo development, cellularisation of endosperm, maximum cross-sectional area of the embryo

sac, chalaza endosperm area, nodule number, mature seed weight[6], classification of mature seed phenotype, and seed viability produced by all FBP mutants studied (*tt4*, *tt5*, *tt6*, *fls*, *tt7*, *tt3*, *tt18/11*, *ban*, *anl1*, *tt19/14*, *tt12*, *tt10*, *aha10* and *ttg2*) is summarised in Table 3.3.

[6] The ratio of a mean weight of mature seed and the percentage of plump and burst seeds were calculated as in section 2.2.4.

Table 3.3: Summary data for seeds derived from crosses between FBP mutants (Col-0 ecotype) and Col4x pollen parents. The mean weight of mature seeds may be one of the more reliable measures to indicate 'rescue' of Col4x-induced seed lethality by crosses involving maternally carried mutations in the FBP. Thus, the series of FBP mutant crosses detailed in this table are ordered by seed weight running from heaviest at the top. Values are described as the mean ± S.E., with sample sizes (**n**) in brackets. The samples size for measurements of embryo sac area in seed development at 5 and 7 DAP was 10 seeds for all mutants except *tt7* at 7 DAP where 8 seeds were sampled. For columns indicating embryo stage, black-shaded cells indicate samples where cellularisation of the peripheral endosperm was observed. **Abbreviations: ***: background ecotypes; **nd**: no data; ◆: no rescue ratio values obtained.

Genotype	Seed development at 5 and 7DAP								Mature seeds				
	Embryo stage		Embryo sac area (µm ²)		Chalazal endosperm area (µm ²)		Number of nodules		Seed weight (µg)	% of 'plump & burst' seed	Weight ratio	'Plump and burst' ratio	Seed viability %
	5 DAP	7 DAP	5 DAP	7 DAP	5 DAP	7 DAP	5 DAP	7 DAP					
Col2xX Col 2x (reference cross)	heart	torpedo	nd	nd	nd	nd	0	0	17± 3 (250)	nd	◆	◆	nd
Col2x *	early heart	overgrown globular retarded	40026 (10)	50771 (10)	2645 (10)	6312 (10)	0.8 (10)	0.5 (10)	14 ± 2 (517)	6 ± 1	◆	◆	7
tt4	early heart	overgrown globular retarded	34534 (10)	130296 (10)	1716 (10)	14663 (10)	1.2 (10)	0.8 (10)	25 ± 1(483)	21 ±2	1.8	3.5	7
aha10	globular	heart	42186 (10)	104745 (10)	1378 (10)	7759 (10)	1.5 (10)	0.6 (10)	24 ± 1 (315)	45 ± 5	1.7	8	19
tt6	heart	heart	38077 (10)	103324 (10)	994 (10)	4604 (10)	0.5 (10)	1.2 (10)	22 ± 1 (243)	27 ±5	1.6	4.5	26
tt12	heart	overgrown globular retarded	43772 (10)	47249 (10)	2069 (10)	4274 (10)	1.3 (10)	1.2 (10)	21 ± 1 (509)	54 ± 8	1.5	9	7
ttg2	early heart	early heart	42343 (10)	100087 (10)	959 (10)	4261 (10)	1 (10)	0.7 (10)	20 ± 2 (389)	28 ± 4	1.4	5	31
tt3	heart	heart	46876 (10)	129727 (10)	1832 (10)	17210 (10)	1.1 (10)	0.4 (10)	19 ± 1 (459)	34 ± 2	1.4	6	17
tt18/11	globular	heart	61442 (10)	72627 (10)	1622 (10)	2775 (10)	0.4 (10)	1 (10)	18 ± 2 (177)	16 ± 4	1.3	3	6
fls	heart	overgrown globular retarded	39521 (10)	52740 (10)	5384 (10)	8939 (10)	0.8 (10)	0.3 (10)	17 ± 1 (272)	19 ± 8	1.2	3.2	7
tt19/14	globular	overgrown globular retarded	23655 (10)	109010 (10)	873 (10)	6471 (10)	0.3 (10)	0.2 (10)	17 ± 2 (245)	20 ± 5	1.2	3.3	5
ban	heart	heart overgrown retarded	40540 (10)	101176 (10)	881 (10)	6430 (10)	1.9 (10)	2.4 (10)	14 ± 1 (178)	18 ± 7	1	3	4
tt7	early heart	underdeveloped & abnormal	56844 (10)	42854 (8)	1866 (10)	1259 (8)	0.8 (10)	1.4 (8)	12 ± 1.2 (340)	21 ± 4	0.9	3.5	8
tt10	globular	overgrown globular retarded	38261 (10)	108284 (10)	1662 (10)	11190 (10)	0.9 (10)	0.1 (10)	12 ± 2 (286)	7 ± 2	1	1.2	3
tt5	heart	overgrown globular retarded	59589 (10)	106769 (10)	2592 (10)	14357 (10)	1.5 (10)	0.8 (10)	10 ± 1 (566)	19 ± 2	0.7	3.2	8
anl1	globular	globular	40931 (10) (10)	80717 (10)	764 (10)	52555 (10)	0.7 (10)	1.9 (10)	6 ± 1 (210)	5 ±2	0.4	0.8	3

3.3 Discussion

A. thaliana displays post-zygotic hybridisation barriers in both interploidy intraspecific and interspecific crosses. The variation of these hybridisation barriers depends on the imbalance of parental genomes when crossing at different ploidy levels. As previously mentioned (section 1.5), hybridisation between a maternal diploid and a tetraploid pollen parent can generate a viable seed. In most *Arabidopsis* ecotypes these seeds typically display delayed endosperm cellularisation and accelerated mitosis resulting in abnormally large but viable mature seeds though the Col-0 ecotype is a notable exception where this paternalisation phenotype is more severe leading to high levels of seed abortion. In contrast, for all *A. thaliana* ecotypes studied, hybrid seed originating from crosses between maternal diploid and hexaploid pollen parents aborts. This seed starts their development with a phenotype similar to that of progeny from 2x X 4x crosses (seed development 5-7 DAP), but when the embryo reaches the globular or heart stage, development is arrested, and unviable seed is produced (Scott *et al.*, 1998). Moreover, the paternal or maternal excess affects the resulting seed phenotype; for example, having paternal excess produces larger seeds than the wild-type, while maternal excess produces small seeds. It is probable that parental imprinting is responsible for this phenomenon (Raissing *et al.*, 2013). In the Col-0 ecotype, maternal excess crosses (4xX2x) result in small but viable seeds. However, paternal excess crosses (2xX4x) produce abnormal large seeds that are mostly non-viable, this variation intolerance is due to endosperm over-proliferation and lack of endosperm cellularisation when the tetraploid partner is Col-0 pollen. Thus, for the Col-0 ecotype, an asymmetric triploid block exists caused by the Col4x lethal sperm effect (Dilkes *et al.*, 2008). The Col-0 ecotype produces pollen/sperm that is especially aggressive in favouring endosperm proliferation and repressing endosperm cellularisation, an effect that has been hypothesised to be due to differences in genomic imprinting of as yet uncharacterised loci in this ecotype (Dilkes *et al.*, 2008; Bolbol, 2010; Kohler *et al.*, 2010).

As mentioned in the introductory section, seeds resulting from crosses between parents of the Col-0 ecotype seem to show a much lower tolerance than the seeds with a parent from the L*er* ecotype in interploidy crosses (Dilkes *et al.*, 2008). Sabelli and Larkins (2009) pointed out that the physical constraints enforced by the seed coat might be weakened by a large multicellular endosperm and that this could restrict the embryo growth. Moreover, this process indicates that an asymmetric hybridisation barrier is

involved. FBP mutants were extensively studied, especially in relation to seed coat phenotypes (Figure 3.21 and more details in Appendix A).

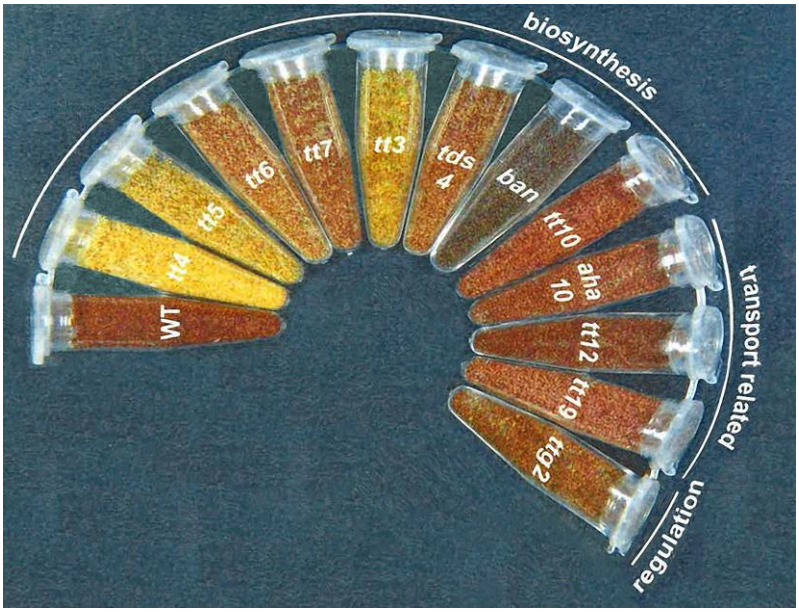


Figure 3.21: Seed colour phenotype of FBP mutants in Col-0. The classification of mutants as presented by Appelhagen *et al.*, (2014) according to functional defects in PAs biosynthesis, transport and regulation (adapted from Appelhagen *et al.*, 2014).

The results collected from the pilot study in this chapter where FBP mutants from different genetic backgrounds were crossed with Col4x pollen parents provides evidence that there is variation in tolerance to Col4x pollen-induced lethality in different ecotypes, as proposed by Dilkes *et al.* (2008). These authors hypothesise that rescue of Col4x induced lethality may be due to a lack of proanthocyanidins (PAs), the final products of the FBP, in the inner integument thus affecting the permeability of this layer which would lead to increased transport of a cellularisation factor from maternal integuments into the endosperm. This would cause an early increased concentration of a cellularising factor, leading to endosperm cellularisation and Col4x-induced lethality being rescued. However, if this hypothesis is correct, then any mutants that disrupt the pathway leading to a reduction or lack of PAs in the inner integuments should relieve Col4x-induced lethality of seeds; however, the results presented in this chapter are frequently not entirely consistent with this hypothesis. For example *ban* mutants generally failed to rescue the high levels of seed lethality observed in Col-0 2x X 4x crosses, despite the fact that this mutation would block the production of PAs in the seed coat. On the other hand *aha10*

mutant lines (which are reported to reduce the levels of PAs 100 fold – Baxter *et al.* 2005) appeared to consistently lessen the effects of Col4x-induced lethality of seeds across a number of measures including seed weight, plumpness and viability. The complexity of the wide range of phenotypes observed for the different crosses makes the formulation of unambiguous hypotheses difficult. However, it seems likely that a simple reduction or block in the production of PAs cannot completely account for the alleviation of Col4x-induced seed lethality. It is likely that a number of the mutations studied would impact on the flux of intermediates in the FBP leading to potential bottlenecks, which in turn could lead to regulatory changes that would be hard to predict.

Flavonol synthase (FLS) catalyses the first step in the flavonol branch of the FBP and blocking the pathway at this point would likely alter pathway flux potentially leading to a large increase in anthocyanin and PAs accumulation. The *fls-1* gene does not rescue Col4x-induced seed lethality and the results were variable between the two experiments conducted with *fls-1* that are presented in this chapter. The first data set produced (pilot study – see section 3.2.1.3.1) for the *fls-1* (EMS) X Col4x cross-resulted in an exceptionally high level of seed lethality and it was not possible to weigh them owing to their fragility. These seeds died prematurely leading to a shrivelled phenotype, which were much smaller than the surviving crosses. However, confocal analysis of seed development in the *fls-1* (EMS) X Col4x cross seemed to indicate that these seeds survived for longer with more intact structures (such as the embryo and chalazal endosperm) as opposed to Col2x X Col4x from which the only identifiable structure at 7DAP is the seed coat (Figure 3.3).

The L*er* ecotype exhibits tolerance to the proliferative effect of Col4x sperm and allows rescue of embryonic lethality, suggesting a stronger maternal effect favouring the micropylar pole, which is powerful enough to compensate for the Col4x paternal effect and restore normal timing of cellularisation (Dilkes *et al.*, 2008). In support of the findings of Scott *et al.* (1998) and Dilkes *et al.* (2008), in this study, the L*er* ecotype generally led to the production of viable seeds in crosses to Col4x pollen, in contrast to the other ecotypes investigated.

The effect of *TT4* mutations in the L*er* ecotype was investigated in the early phases of this study - *TT4* acts at the first stage of the FBP and encodes chalcone synthase (CHS) (Figure 1.9), and, therefore, its disruption should, in theory, have a substantial effect on

the FBP. *tt4-1* (L*er*) X Col4x crosses generally showed rescue of Col4x-induced seed lethality when taking multiple phenotypic characters together. However, individual classes of data such as the percentage of plump and burst seed (Figure 3.10) do not provide substantial evidence for a rescue effect. Interestingly the *ttg2-1* mutant line (a major transcription regulator of the FBP) had a slightly better rescue effect than *tt4* despite the fact that the seeds of *ttg2-1* lines are darker in colour than *tt4*.

As mentioned above multiple steps of the FBP are regulated by TTG2, a WRKY transcription factor that is expressed in the seed endothelium; and which is encoded by TTG2. Mutation of *ttg2-1* (L*er*) results in disruption of the FBP, which in turn, according to the model, prevents inhibition of the maternal cellularisation signal (Figure 1.13). Consequently, *ttg2-1* mutants rescued Col4x-induced embryonic lethality by restoring the normal timing of cellularisation. The strong rescue of *ttg2-1* (L*er*) mutants indicates a key role of the transcription factor in promoting gene expression of key components of the FBP, since mutation of *ttg2-1* compromises the FBP enough to overcome the powerful Col4x paternal effect (Dilkes *et al.,* 2008). Study of the SALK_148838.51.70 T-DNA insertion in *TTG2* in the Col-0 ecotype (part of the major study presented in this chapter) confirmed the strong effect mutation of this gene could have on rescuing Col4x induced lethality.

Analysis of mutants for the *TT10*, the last gene of the FBP, whose product operates inside the vacuole of the endothelium layer provided useful information on the potential role of PAs in the rescue of Col4x-induced seed lethality. TT10 has a role in the oxidation of PAs (David *et al.*, 2014) and catalyses the change to PAs from procyanidin (Figure 1.9). Mutation of TT10 in both the L*er* and Col-0 ecotypes failed to rescue Col4x-induced seed lethality (Figure 3.9). Indeed, the *tt10-5* (L*er*) X Col4x crosses appeared to have a slight negative effect on seed development compared to the control whereas in the *tt10* (Col-0) X 4x crosses no significant difference from the Col 2x X 4x cross was evident. The inability of *tt10* mutants to rescue Col4x lethality would suggest that conversion of colourless PAs to their oxidised form it is not a key step in the inhibition of the maternal cellularisation signal (Figure 1.9). This implies that the maternal cellularisation signal is probably inhibited by an earlier metabolite or product of the pathway, or that there is an alternative mechanism for the production of oxidised PAs production in the vacuole that complements the *tt10-5* to allow inhibition of cellularisation and seed lethality.

TT12 encodes a key proton-epicatechin antiporter, which is necessary for the transport of epicatechin into the vacuole. Mutation of this transporter prevents accumulation of PAs in the vacuoles, and therefore, it prevents the FBP from inhibiting the maternal cellularisation signal. Assessment of *tt12* mutant lines in both the Col-0 and Ws ecotypes for their ability to rescue Col4x-induced seed lethality indicated that seeds showed multiple characteristics indicative of a strong rescue effect. Even though *tt12* lines rescue seed lethality in these crosses Ws and Col-0 mutants still exhibited delayed cellularisation relative to what is expected for normal seed development (Figure 3.1) as can be seen by the phenotype of the *tt12-1* (Ws) X Col4x crosses (Figure 3.9). This may be attributable to alternative mechanisms for the transport of anthocyanin into the vacuole or that components of the FBP are only one factor that influences regulation of endosperm proliferation and cellularisation. *TT19*/*14* has been suggested to play a role in an alternative route for the import of anthocyanins. This redundancy could explain that whilst *tt12* mutants rescued seed lethality, they did not show wild-type timing of cellularisation due to an alternate mechanism, allowing a low level of PAs accumulation in the vacuole which may partially inhibit the maternal cellularisation signal (Zhao *et al.*, 2010; Frank *et al.*, 2011).

TT16 encodes a transcription factor involved in the regulation of the FBP and mutation of this gene in the Ws ecotype was investigated in the pilot study only. *TT16* does not play as important a role in regulation of gene expression across the FBP as *ttg2-1* does, and may control expression of redundant enzyme isoforms or influence expression of the BAN gene that, as has been shown in this study, does not rescue seed lethality when mutated (Zhao *et al.*, 2010). *tt16* (Ws) X Col4x crosses did not show rescue of lethality. By 9 DAP the embryo is arrested at the globular stage of development and the structures seem fragmented and ill-defined with many nodules, whereas the *tt12-1* (Ws) X Col4x achieved cellularisation by this stage. This can be seen more clearly in the images of mature seeds (Figure 3.9.G, H and I), which show that *tt12-1* (Ws) X Col4x crosses producing larger seeds with a more normal plump phenotype, whereas the *tt16* (Ws) X Col4x produced seeds that were largely lethal having a highly shrivelled phenotype.

Normal development of *A. thaliana* seeds requires that endosperm proliferation occurs relatively early to result in a large seed cavity area and this should be followed by endosperm cellularisation. The embryo then proceeds to grow in the cavity area replacing the endosperm as development progresses (Sundaresan, 2005; Berger *et al.*, 2006; Sabelli

and Larkins, 2009). As mentioned previously, the Col-0 ecotype is intolerant to genomic imbalance; crossing with a paternal Col4x (paternal excess) leads to triploid block via aberrant seed development (Dilkes *et al.*, 2008). Paternal excess results in high endosperm proliferation characterised by a large chalaza, delay or absence of endosperm cellularisation and inhibition of normal embryo development.

In this study most FPB mutants in the Col-0 ecotype failed to rescue Col4x-induced seed lethality, at least in terms of producing high percentages of plump viable seeds. However, taking many features of seed development and morphology into consideration it is clear that mutations of components of the FBP pathway did frequently result in various degrees of 'rescue' that is consistent with data obtained for other ecotypes. Clearly the Col-0 ecotype is highly sensitive to the effects of paternal excess and FBP mutations that affect the good rescue in ecotypes. For example Ler ecotype is less effective than Col-0 where it appears that a higher threshold must be reached to attain rescue. Thus, mutations that have a positive effect on rescuing Col4x induced lethality have more subtle 'rescue' phenotypes in this ecotype.

The formation of large chalazal nodules on the peripheral endosperm that lines the central region of the embryo sac limits the space available for embryo growth. Embryogenesis in some of the Col-0 *tt* mutants studied never passed the globular stage. In normal 2x X 2x balanced crosses, endosperm cellularisation occurs early at 5DAP, but in interploidy crosses (Col-0 2x X Col4x), including those harbouring *tt* mutants the endosperm is at the proliferation stage and cellularisation fails to occur by 5DAP. The development of the endosperm at this stage is characterised by hyperplasia (excessive cell growth later in life). The central peripheral endosperm may also become convoluted, possibly to accommodate the large number of protoplasts that have formed (Scott *et al.*, 1998). Endosperm cellularisation, which should have been achieved by 7DAP, was noted to be lacking in seeds that had an increase in their overall dimensions and the size of the chalaza. This was evident in the Col2x X Col4x, *ban* (Col-0) X Col4x and *tt10* (Col-0) X Col4x images. Consequently, it is probable that late endosperm cellularisation, which is linked to seed abortion, was occurring in all seed crosses. By 7DAP most crosses involving FBP mutants in Col-0 ecotypes produced seeds that were significantly different to the control cross, extending dramatically such as *tt4, tt6, tt3, tt18/11, ban, anl1, tt19/14, tt10, aha10* and *ttg2* that was an evidence of the paternal excess effect (Figure 3.12.B).

By 9DAP for the *ban* (Col-0) X Col4x crosses, it was exceedingly difficult to obtain good quality images by confocal laser microscopy as the majority of the seeds had collapsed compared to the control cross (Figure 3.13).

So, based on the above data, it would be possible to agree with Dilkes *et al.* (2008) that there is no correlation between the area of the embryo sac and the rescue ratio. Nor is there a correlation between the area of chalazal endosperm and seed survival according to Dilkes *et al.* (2008) - the only characteristic that typically correlated with improved seed survival was earlier endosperm cellularisation (Dilkes *et al.*, 2008). From the work presented here there is no evidence from the data collected for the Col-0 ecotype to suggest that mutants that effect 'rescue' have a reduced embryo sac area – as mentioned above due to the high sensitivity of Col-0 to paternal excess and the attenuated 'rescue' phenotypes observed (seeds from all crosses suffered from the effects of paternal excess) it is hard assessed precisely what factors were providing 'rescue' in these crosses. The main hypothesis that rescue of Col4x-induced seed lethality is effected by timely cellularisation of endosperm (Dilkes *et al.*, 2008) remains a plausible mechanism and is supported by some of the data presented in this study. In most of the crosses studied for the *tt* mutants in the Col-0 ecotype evidence of cellularisation was not found by 7DAP and thus, further work will be required in the future to clarify if and when cellularisation occurs in these crosses.

A. thaliana seeds with double paternal genome contributions to the seed display accelerated endosperm mitosis and delayed or failed endosperm cellularisation. As Scott *et al.* (1998) stated, mature seed size increases as genome number increases; however, changing the parental genome ratio has an even bigger effect on the size of seeds and their dry weight. It has been suggested that seed weight and embryo size are correlated to endosperm proliferation (Scott *et al.*, 1998; Schruff *et al.*, 2006).

In 2x X 4x interploidy crosses, the seed has an embryo (3x) containing twice the normal number of paternal genomes (1m: 2p). The endosperm resulting from these crosses (4x) is affected by paternal genome dosage (2m: 2p) and this leads to abnormal seed development. This is characterised by accelerated endosperm mitosis and a delay in, or failure of, endosperm cellularisation - this excessive over-proliferation is linked to embryo abortion (Scott *et al.*, 1998). The crosses that rescued the seed lethality (in L*er* and Ws ecotypes, but not in the Col-0 ecotype), produced seeds that were significantly

heavier than the crosses that were lethal and this is associated with cellularisation occurring at a point that prevents seed collapse.

Measurements for the numbers of burst and plump mature seeds and the weight of mature dry seeds were used to calculate 'rescue ratios'. Taking all the data into consideration seed dry weight gave a more reliable indicator of 'rescue' for most crosses and thus, the discussion will focus on the ratios between the mature seed weights of both mutant and WT for the study in the Col-0 ecotype. Interestingly, the cross that showed the greatest rescue ratio for dry weight seed was the tt4 X Col4x cross. *TT4* encodes chalcone synthase and is the first step in the pathway so rescue was anticipated – *TT4* knockout in the L*er* ecotype also affected a rescue phenotype. The next step in the pathway is regulated by *TT5* and it would be expected that knockout of this gene would also bring about a rescue of Col4x-induced lethality however *tt5* failed to rescue. An explanation for this is that the *tt5* allele used is almost certainly a knockdown rather than knockout allele and even though these seeds are pale in colour (pale brown rather than yellow) the pathway apparently still operates to a degree that permits highly abnormal seed development.

Importantly the *tt6* mutant was able to effect good 'rescue' and this encodes F3H, the next enzyme in the FBP. The data for *tt7* (the next step in the pathway) was somewhat mixed with evidence of 'rescue' in the ratios of plump and burst seeds compared to the Col2x X Col4x control. However, estimates of rescue based on seed dry weight was less reliable. Mutation of TT7 would likely block the pathway and thus prevent accumulation of PAs though *tt7* seeds are widely reported as being of a light brown colour (Table 3.2; Debeaujon *et al.*, 2000). Furthermore, perturbation of TT7 may also increase the flux of pathway intermediates into the flavonol branch of the FBP that may impact on aspects of seed development (flavonols are known to be important factors in the regulation of auxin transport). Thus, the seed phenotypes observed in crosses involving *tt7* highlight the potential complexities in understanding the impact of FBP mutations on seed development.

Mutations in *TT12* were particularly effective at rescuing Col4x-induced seed lethality in both the Col-0 and Ws ecotypes. *TT12* encodes a vacuolar flavonoid/H+ -antiporter that transports epicatechin into the vacuoles of the seed endothelium – it is noteworthy that mutations in *AHA10* that encodes a vacuolar H+-ATPase also affected good 'rescue' in Col-0. Both *aha10* and *tt12* seeds have abnormal vacuolar morphology and reduced levels

of PAs (Baxter *et al.*, 2005; Pourcel *et al.*, 2005; Zhao *et al.*, 2010) suggesting that either PAs and/or normal vacuolar morphology in the endothelium are important factors in the regulation of seed development. In contrast, *fls-1* X Col4x and *anl1* (Col-0) X Col4x had the lightest mature seed rescue ratios from the two studies reported in this chapter. Mutation of *ANL1* is likely to increase flux in the pathway towards PAs biosynthesis via epicatechin thus reinforcing late cellularisation of developing endosperm. Similarly, mutation of *FLS* may have the same effect, directing more pathway intermediates to PAs biosynthesis as FLS regulates entry into the flavonol branch of the FBP (see Figure 1.9).

One perplexing result was the ineffectiveness of *ban* mutants at overcoming Col4x-induced seed lethality given its position in the FBP. *ban* mutants are defective in the conversion of cyanidin to epicatechin and the affected regulatory enzyme is anthocyanidin reductase (ANR). Most of the quantitative data suggested that the outcome of crosses involving *ban* were very similar to the control Col2x X Col4x cross. However, it is worth noting that the T-DNA insertion in *ban* is located in the promoter region of the gene and although a strong *ban* phenotype was evident in this line (accumulation of anthocyanin in the seed coat) some transcript was detected by RT-PCR (Figure 3.11). Thus, it cannot be ruled out that the *ban* mutant produces enough of a downstream factor that is responsible for seed lethality.

Overall following interploidy crosses, seed survival is strongly affected by genetic ecotype in *A. thaliana* as well as being influenced by the FBP – indeed, the L*er* ecotype is known to have reduced expression of *TTG2* (Dilkes *et al.*, 2008). In addition, the results showed that FBP mutants in Col-0 ecotype crossed with Col4x had relatively low mature seed weights despite the fact that the seed cavity was observed to be much larger than controls. The likely reason for this apparent discrepancy is that the number of endosperm nuclei increased dramatically during seed development in these crosses. However, the low level of endosperm cellularisation resulted in a failure of endosperm to feed the growing embryo sufficiently in many seeds and subsequently these seeds collapse impacting on the mean seed weight values.

So, based on the above discussion, there needs to be further investigation to identify the signalling molecule(s) that are crucial for in endosperm cellularisation and factors that influence the movement of the cellularisation 'signal'. Importantly a role for auxin should be considered, as certain flavonols are known to be important in regulating auxin

transport. Further auxin is known to be vital in regulating plant growth generally as well as having an important role in the growth of seeds (Locascio *et al.*, 2014). Additional information about the relevance of auxin in this project will be presented in Chapter 6. Also, the cellularisation factor that rescues Col4x-induced lethality might, indeed, be discovered following further analysis of the FBP.

Finally, the results of rescue and lethal phenotypes arising from interploidy crosses in L*er*, Ws and Col-0 ecotypes, from this project and previous work (Scott, pers. comm.) are summarised in figure 3.22.

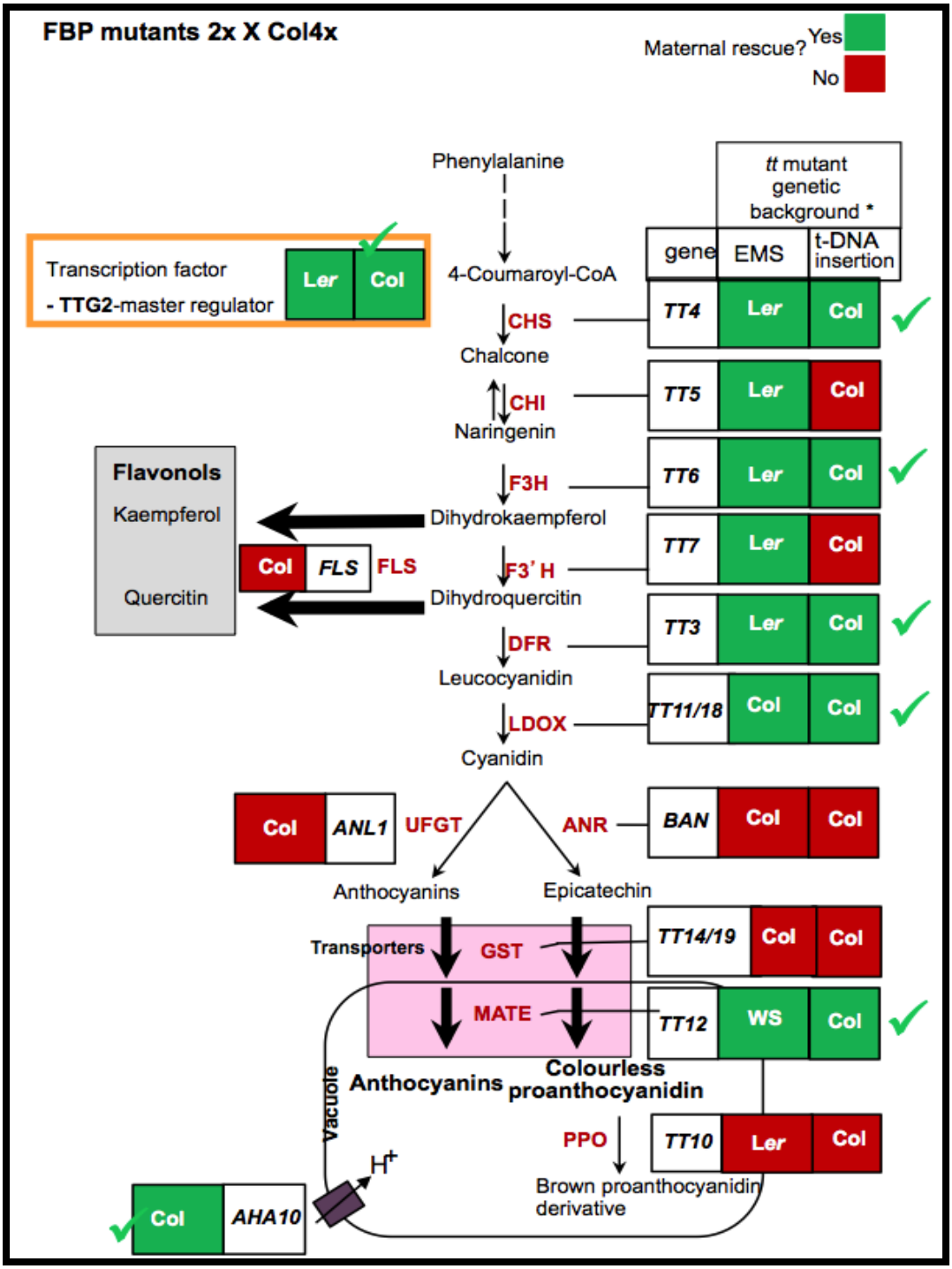


Figure 3.22: An updated model was summarising the effects of FBP mutants in crosses to Col4x in *A. thaliana* seeds. Enzymes are shown in a red font. Mutant alleles of genes with green backgrounds rescue Col4x induced seed lethality (paternal excess). Genes with red backgrounds do not rescue seed lethality. The presence of an active pathway is proposed to disrupt the transport of a cellularisation factor into the endosperm. *TT12* and *AHA10* are involved in correct vacuole biogenesis. Asterisk indicates mutations that were generated by either ethyl- methanesulfonate (EMS) or T-DNA insertional mutagenesis in the L*er*, WS and Col-0 *A. thaliana* ecotypes. Rod Scott provided some data for EMS mutants. The FBP pathway figure is modified from that of Winkel-Shirley, 2001; Debeaujon *et al.*, 2003; Baxter *et al.*, 2005; Pourcel *et al.*, 2005; Lepiniec *et al.*, 2006; Kleindt *et al.*, 2010 and Appelhagen *et al.*, 2014; Scott pers. comm.).

3.4 Summary and conclusions

Arabidopsis Col4x, when acting as a pollen parent usually yields large seeds that suffer high levels of developmental lethality when crossed with diploid mothers. Both endosperm differentiation and many aspects of the endosperm cell cycle are affected by the parental genome dosage present in these *A. thaliana* seeds. Mature seed size is significantly affected by the relative paternal and maternal contributions and from this, we can infer that the ability of the endosperm to obtain resources from the seed parent is likewise affected.

Seed derived from most crosses between FBP mutants (Col-0) and Col4x have an abnormal pattern of development due to the powerful paternalising effect of Col4x sperm in the endosperm of the Col-0 ecotype. Despite the overall weakness of seed rescue seen in Col-0 compared to other *Arabidopsis* ecotypes trends in 'rescue' were evident. The data obtained suggested that mutations early in the FBP and those affecting vacuolar biogenesis and PA accumulation went some way to ameliorating Col4x-induced lethality. Further genetic dissection of the pathway would likely provide valuable information on the essential products of the FBP that are important in regulating cellularisation of endosperm. Therefore, work presented in the next chapter will explore the utility of using double FBP mutants in the Col-0 ecotype in an attempt to further understand the role of the particular products of the FBP in the regulation of seed development.

Chapter 4: Generating and evaluating Col-0 FBP double mutants to further understand a potential role for flavonoids in mediating seed development in *A. thaliana*

4.1 Introduction

The work presented in Chapter 3 investigated Col4x-induced seed lethality in Col-0 and other several other ecotypes and included an extensive investigation into the role of various FBP mutants in alleviating this lethality. This included using crosses between maternally carried single mutants in the FBP and Col4x pollen parents. Importantly the analysis revealed that some mutants were capable of 'rescue' of Col4x-induced seed lethality and these lines generally had a higher percentage of plump and burst rescue seeds than the control Col2x X Col4x. This genetic dissection of the FBP highlighted that mutations in enzymes that act early in the pathway generally lead to 'rescue' of Col4x-induced seed lethality. However, the data could not permit identification of a particular intermediate or end product of the pathway that, once removed by mutation, was responsible for the rescue effect.

This chapter extends the work reported in Chapter 3 and reports on experiments designed to investigate the potential role of flavonoids (section 1.6.2) by using double mutants for genes of the FBP as seed parents in the interploidy crosses with Col4x pollen parents. Using double mutants would permit blocking of the FBP at separate points to significantly alter flux within the pathway in the anticipation that more precise predictions can be made about the roles of FBP intermediates and products in blocking or promoting endosperm cellularisation. The double mutants used in this study were *anl1/ban, tt12/anl1*; *ban/tt12*.

ban mutants do not accumulate proanthocyanidins (PAs) in seeds but instead are characterised by having high accumulations of anthocyanins (Abrahams *et al.*,2002). By combining the *anl1*, which affects the later stages of anthocyanin biosynthesis and accumulation (Kubo *et al.,* 2007), with *ban*, a block (or at least a severe decrease) of anthocyanin and PAs accumulation in the vacuole of the endothelium should be achieved. Furthermore, this may lead to a build-up of pathway intermediates upstream of *ban* and *anl1*, in particular, cyanidin.

TT12 is a tonoplast MATE family transporter (acts as a proton antiporter) responsible for the import of epicatechin and anthocyanins into the vacuole of seed endothelial cells

(Marinova *et al.*, 2007) and thus, combination of *tt12* lines with either *ban* or *anl1* should lead to the accumulation of anthocyanins and epicatechin in the endothelium cytoplasm respectively (Petrussa *et al.*, 2013) (see Figure 1.9).

It is noteworthy that attempts were made to generate an *anl1*/*fls* double mutant in order to block flavonol (principally kaempferol and quercetin) and anthocyanin production while retaining production of PAs. Unfortunately homozygous double mutants could not be recovered suggesting that this combination of FBP mutations has a lethal effect on seed development.

4.1.1 Aims and objectives

The aims of this chapter were to:

1. Utilise a reverse genetics approach – by generating *tt* double mutants lines - to investigate what intermediates/products of the FBP play a role in modulating Col4x-induced seed lethality;
2. Test whether it is possible to alter the flux and accumulation of flavonoid metabolites, to enhance 'rescue' of Col4x induced seed lethality via alteration of the timing of endosperm cellularisation;

4.2 Results

4.2.1 Preliminary phenotypic analysis of double mutants

Seed coat colour of the double mutants for mature seeds was observed (Figure 4.1).

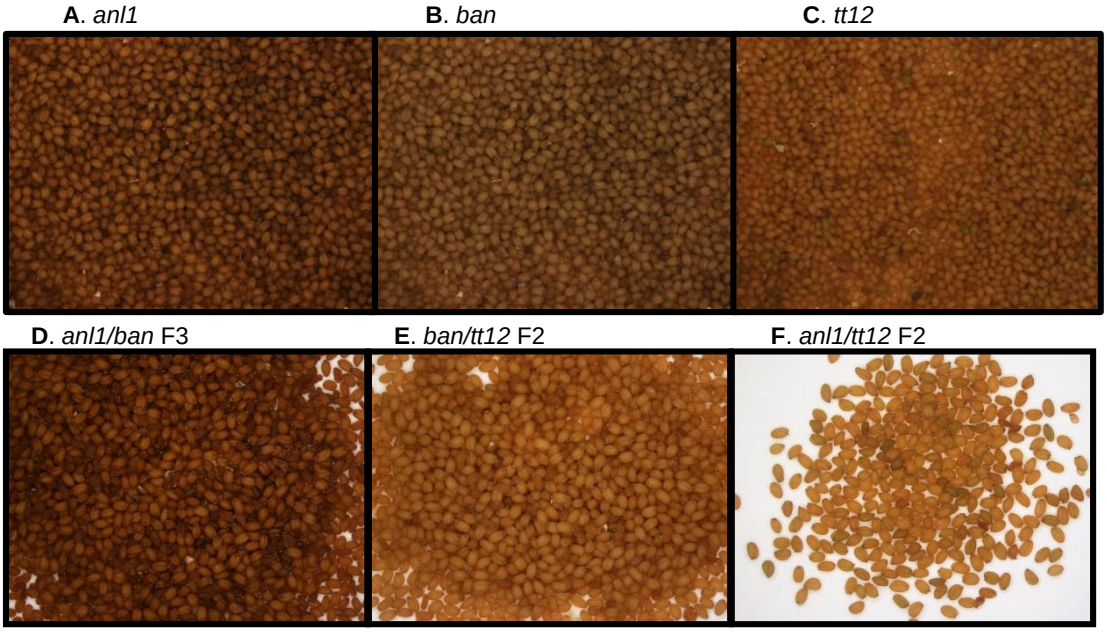


Figure 4.1: Accumulation of pigments in mature seeds of FBP double mutants. All seeds are the result of self-pollination of each single and double mutant line. *anl1/ban* seeds were mostly brown / plump (93%) or dark brown/ shrivelled (6%). The majority of *ban/ tt12* seeds were pale brown/ plump (96 %) with a few being tan brown/ large and shrivelled (4%). 77 % of pale brown/plump and dark brown/shrivelled approximately 22 % in *tt12/ anl1* seeds.

4.2.2 Developmental analysis of seeds resulting from crosses between a selection of FBP double mutants and Col4x pollen parents

4.2.2.1 Analysis of crosses involving anl1/ban, tt12/anl1, and ban/tt12 FBP mutants

Results showed a well-defined difference between the seeds derived from FBP double mutant crosses and the control Col2x X Col4x. Embryos in seeds from *anl1/ban, tt12/anl1, ban/tt12* and *anl1* reached the globular stage by 5DAP whereas seeds resulting from the control Col2x contained early heart stage embryos, as did those from the *ban* and *tt12* (Figures 4.2.A-B). Moreover, a significant delay in seed development was evident by 7DAP for Col2x, *anl1, tt12, anl1/ban* and *tt12/anl1*, with embryos having an overgrown, abnormal globular-like structure; in contrast, *ban/tt12* embryos continued relatively normal development reaching the heart stage.

At 5 DAP, the central peripheral endosperm had an accelerated mitosis and a delay in endosperm cellularisation. The size of the chalazal endosperm increased in all crosses and some of these became vacuolated. Several *tt* mutants (*ban, tt12* and *anl1/ban*) had an early collapse of the chalazal endosperm and formed chalazal nodules in 5 and 7 DAP seeds.

To investigate this more, a germination test will have to be made.

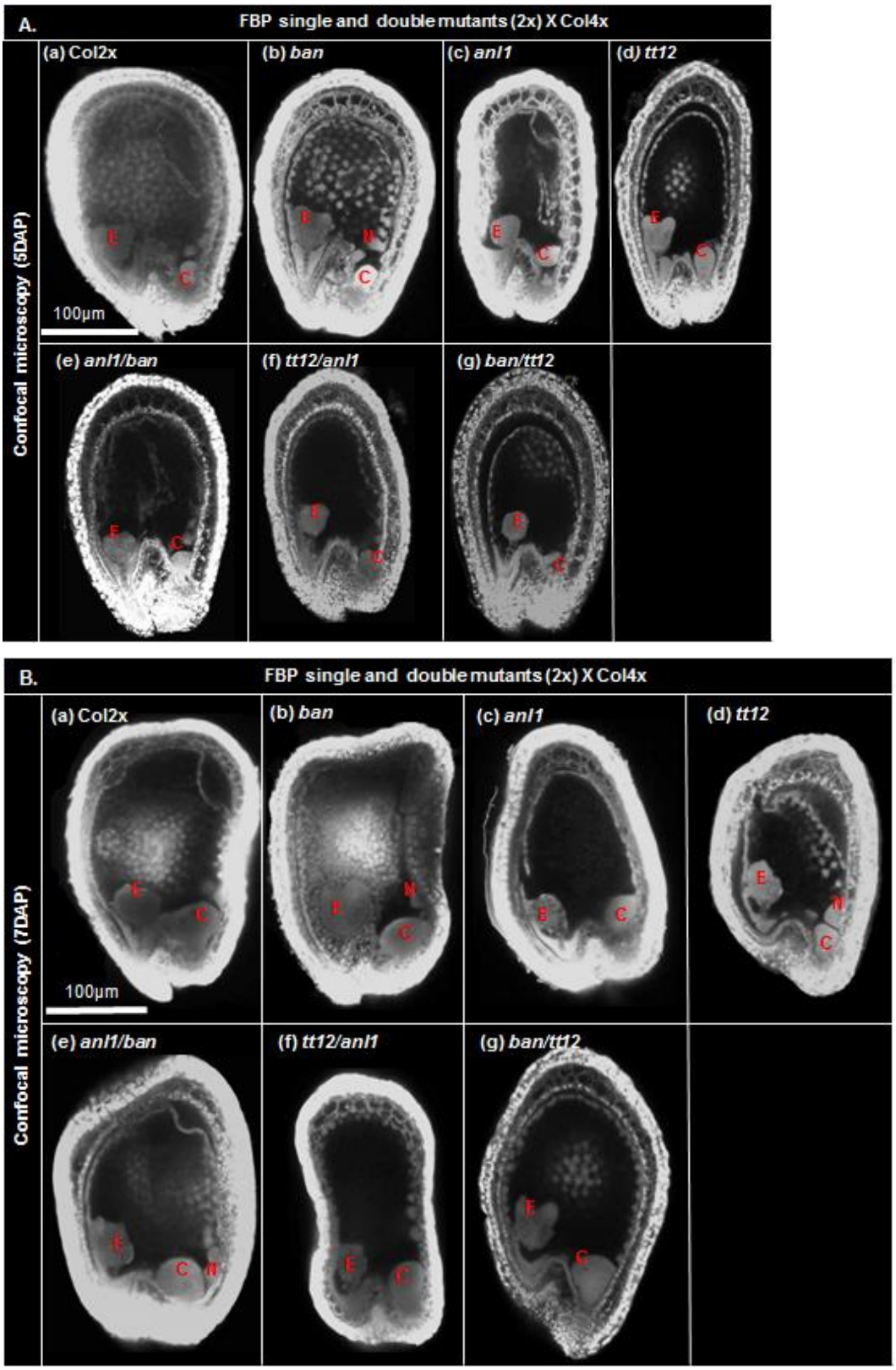


Figure 4.2: Developmental analysis of seeds generated from FBP double mutants crossed to Col4x pollen parents at 5 and 7 DAP. A. Confocal laser-scanning photomicrographs of seeds derived from crosses involving Col-0 FBP double mutants and their single mutants at 5DAP. **B.** Confocal laser-scanning photomicrographs of seeds obtained from Col-0 FBP double mutants and their single mutants at 7DAP. (**a**) Col2x X Col4x control cross, (**b**) to (**d**) FBP single mutants in the Col-0 ecotype crossed to Col4x pollen parents, while (**e**) to (**g**) are Col-0 FBP double mutants crossed to Col4x pollen parents. **E**, embryo; **C**, chalazal endosperm; **N**, nodules. All images were taken at a magnification at 20x. Scale bar = 100µm in all images.

4.2.2.2 Seeds resulting from crosses between double FBP mutants and Col4x pollen parents show a substantial increase in embryo sac area

During seed development, the embryo sac area in seeds resulting from FBP double mutants X Col4x extends dramatically compared to the control Col2x X Col4x cross (Figure 4.3). Results showed that across all lines tested (including controls) *tt12/anl1* X Col4x had the smallest embryo sac area at 5DAP (33054 µm^2), and that *anl1/ban* X Col4x had the largest embryo sac area at 5DAP (47056 µm^2). At 7DAP, *ban/tt12* X Col4x had the greatest embryo sac area (119737 µm^2), while *anl1/ban* X Col4x had the lowest embryo sac area (77185 µm^2).

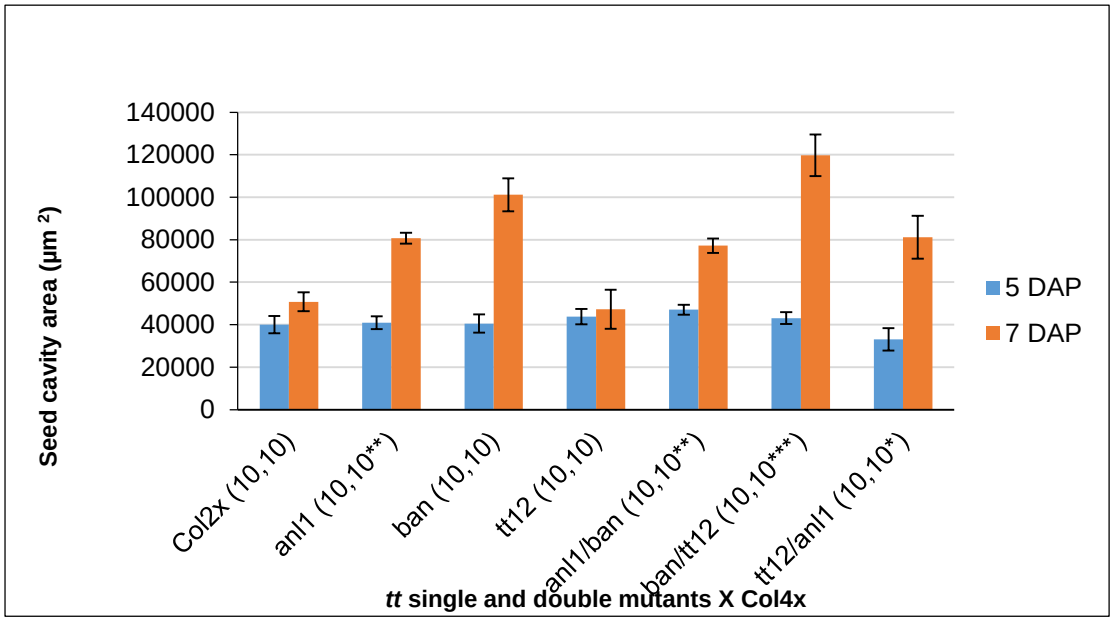


Figure 4.3: Mean values of seed cavity area in seeds resulting from crosses between maternal FBP double mutants and paternal Col4x at 5 and 7 DAP. Embryo sac area seeds are represented using blue and orange bars to denote 5 and 7DAP, respectively. The growth of embryo sac area in FBP double mutants with paternal Col4x seeds increased with seed development. Confocal micrograph images captured at 20x magnification. Error bars represent standard error. Sample sizes are ten seeds. Significant differences were shown by a Mann-Whitney test: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$.

Amongst the double mutants with *ban/tt12* X Col4x seeds, *ban/tt12* X Col4x seeds had the largest embryo sac area across all samples (Figure 4.3). The relatively small embryo sac area recorded at 7DAP for *tt12* was not maintained when this mutation was combined with either *ban* or *anl1* suggesting that the effects of these mutations override the ability of *tt12* lines to restrict embryo sac expansion (See table 4.1 in Appendix B).

4.2.2.3 Seeds resulting from crosses between FBP double mutants and Col4x pollen parents show altered chalazal endosperm areas

Results showed that the area of chalazal endosperm increasing with seed development stages in all crosses (Figure 4.4). At 5DAP, the chalazal endosperm area in the Col2x control cross was the largest, with a mean of 2645 µm^2 but all other crosses involving single or double FBP mutants had reduced chalazal areas at this stage of development. By 7DAP, the chalazal endosperm area in seeds derived from *ban/tt12* cross was the largest, with a mean of 12119 µm^2 that from *tt12/anl1* was the smallest with mean of 4232 µm^2. Apart from the dramatic increase in chalazal area for *ban/tt12* at 7DAP, the general trend was that double mutants had similar or smaller chalazal areas to the single mutants and control at this stage of development (See table 4.2 in Appendix B).

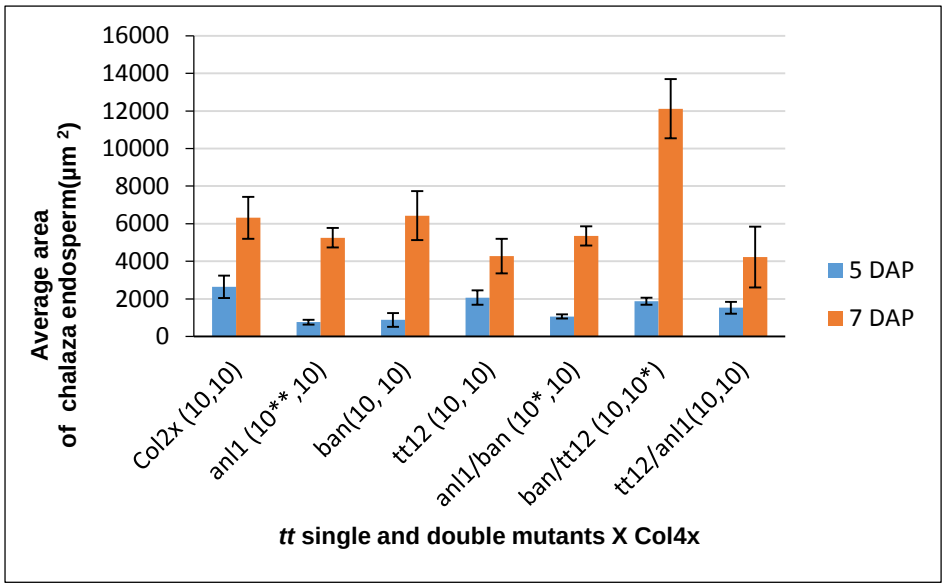


Figure 4.4: Mean chalazal endosperm areas for seeds derived from FBP single and double mutant crossed with Col4x pollen parents. Data is presented for both 5 and 7 DAP. Chalazal endosperm area at 5 DAP are indicated by blue bars and 7DAP by orange bars. In all cases, chalazal endosperm area increases between 5 and 7DAP. Data was obtained from analysis of confocal microscope images taken at 20x magnification. Error bars represent standard error. Sample sizes are ten seeds. Significant differences were shown by a Mann-Whitney test: *, $P \leq 0.05$; **, $P \leq 0.01$.

4.2.2.4 Variation in endosperm nodule between FBP mutant lines and over seed development

Results showed that the average number of nodules varied widely between lines and also frequently between developmental stages (Figure 4.5). At 5 DAP, seeds arising from *ban* X Col4x, *ban/tt12* X Col4x had the highest number of nodules (1.9) and (1.7) respectively

while seeds resulting from *anl1*, *tt12/anl1* crossed to Col4x had the lowest number of nodules (0.7) and (0.8), respectively.

By 7DAP, seeds arising from the double mutant lines *anl1/ban* crossed to Col4x had the highest number of nodules (3.7), whilst seeds from *ban/tt12* and *tt12/anl1* had the lowest nodules number (0.6 and 1.1 respectively). Thus, nodule number is highly variable between lines and over development though double mutants carrying a *tt12* allele do generally appear to have restricted nodule numbers compared to other mutants studied (See table 4.3 in Appendix B).

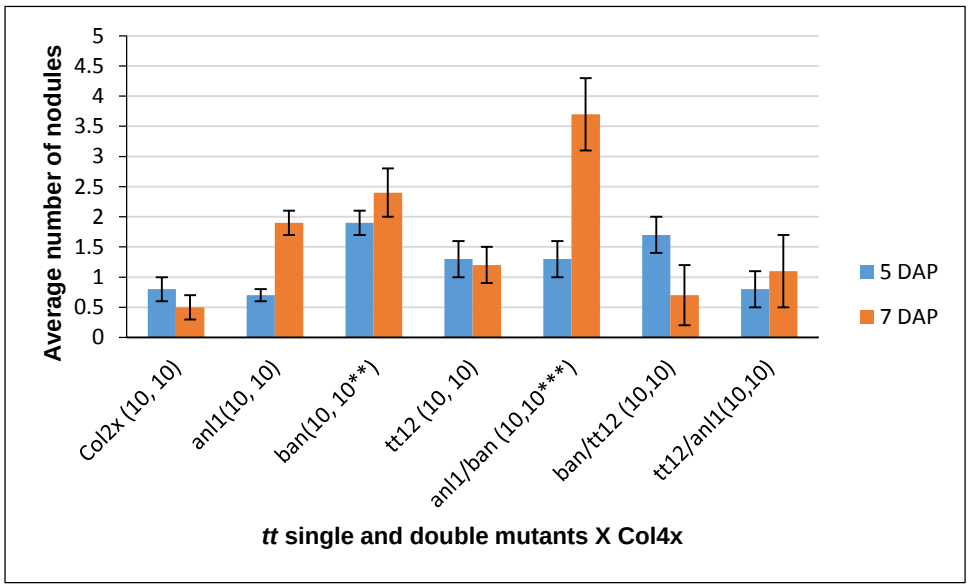


Figure 4.5: Mean number of nodules in seeds derived from FBP double mutants crossed to Col4x pollen parents at 5 and 7 DAP. Blue bars represent data at 5 DAP and orange bars at 7DAP. Mean numbers of nodules were estimated from confocal micrograph images captured at 20x magnification. Error bars represent standard error. Sample sizes are ten seeds. Significant differences were shown by a Mann-Whitney test: **, $P \leq 0.01$; ***, $P \leq 0.005$.

4.2.3 Analysis of mature seeds derived from crosses between FBP double mutants and Col4x pollen parents

4.2.3.1 Analysis of seed weight

Results showed substantial variation in seed weight for seeds derived from the FBP double mutant parents with those from *ban/tt12* X Col4x being the heaviest (Figure 4.6). It is noteworthy that crosses involving *tt12* (21µg) as a single mutant or combined with

ban (25µg) or *anl1* (18µg) as a double mutant generated the heaviest seeds amongst the crosses tested. Seeds derived from the *anl1* X Col4x cross were by far the lightest (6 µg) but interestingly in combination with *ban* or *tt12* as double mutants generated seeds similar in weight to the control Col2x X Col4x cross (See table 4.4 in Appendix B).

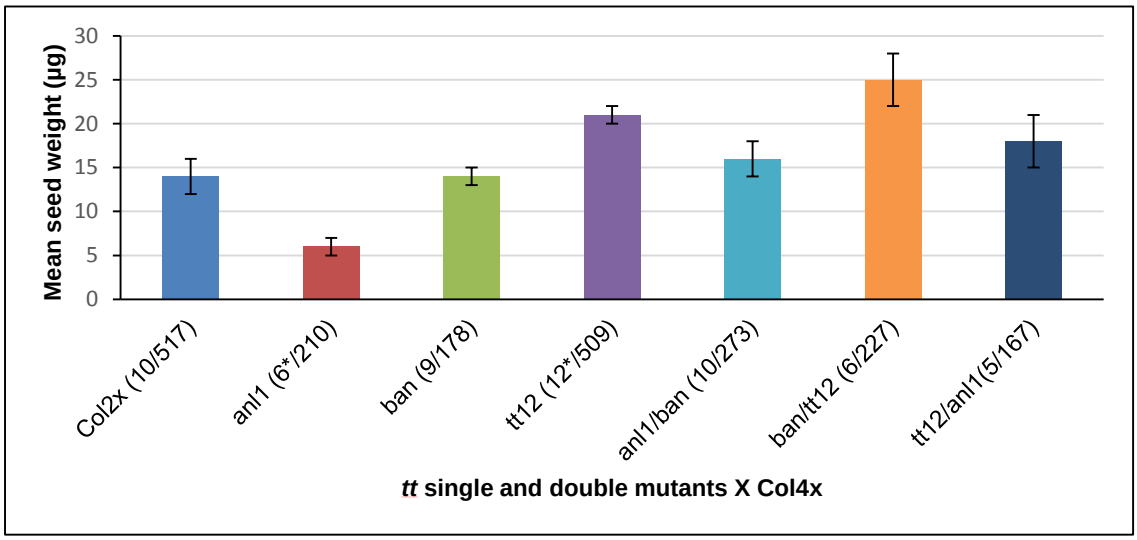


Figure 4.6: Mean weights for mature seeds derived from crosses between FBP double mutants and Col4x pollen parents. Error bars represent standard error. In brackets - (number of siliques/ seed sample size). Significant differences were shown by a Mann-Whitney test: *, $P \leq 0.05$.

4.2.3.2 Effect of double mutants of the FBP on Col4x induced seed lethality – estimation of 'rescue' by utilising (plump and burst) vs. lethal (shrivelled) ratios

Morphological analysis of mature dry seeds showed that most seeds resulting from crosses between the FBP double mutants and Col4x had an irregular shape, with different proportions of 'plump and burst' seeds (large seed size), and 'shrivelled' seed categories (small seed size) (Figure 4.7). The seeds derived from each cross were analysed by calculating the numbers of a) 'plumb and burst' seeds (considered 'rescue' or potentially viable) and b) 'shrivelled' seeds (a developmentally lethal seed and probably unviable) according to their phenotype (illustrated by arrows in Figure 4.7).

Mature seeds derived from *anl1*, *ban* and *anl1/ ban* crossed to Col4x pollen parents showed significantly different ratios as compared to the control cross (Col2x X Col4x) with many more seeds being smaller and irregularly shaped (high proportions of 'shrivelled' seeds) (Figure 4.7 (B), (C) and (E) and Figure 4.8).

Moreover, seeds resulting from *ban/tt12* and *tt12/anl1* X Col4x were larger than control Col2x X Col4x, and had a high proportion of irregular 'plump and burst' seeds (Figure 4.7 (F) and (G) and Figure 4.8). Interestingly, seeds from *ban/tt12* and *tt12/anl1* X Col4x

were noticeably more angular and larger than Col2x X Col4x seeds, and most fell into the 'plump and burst' category.

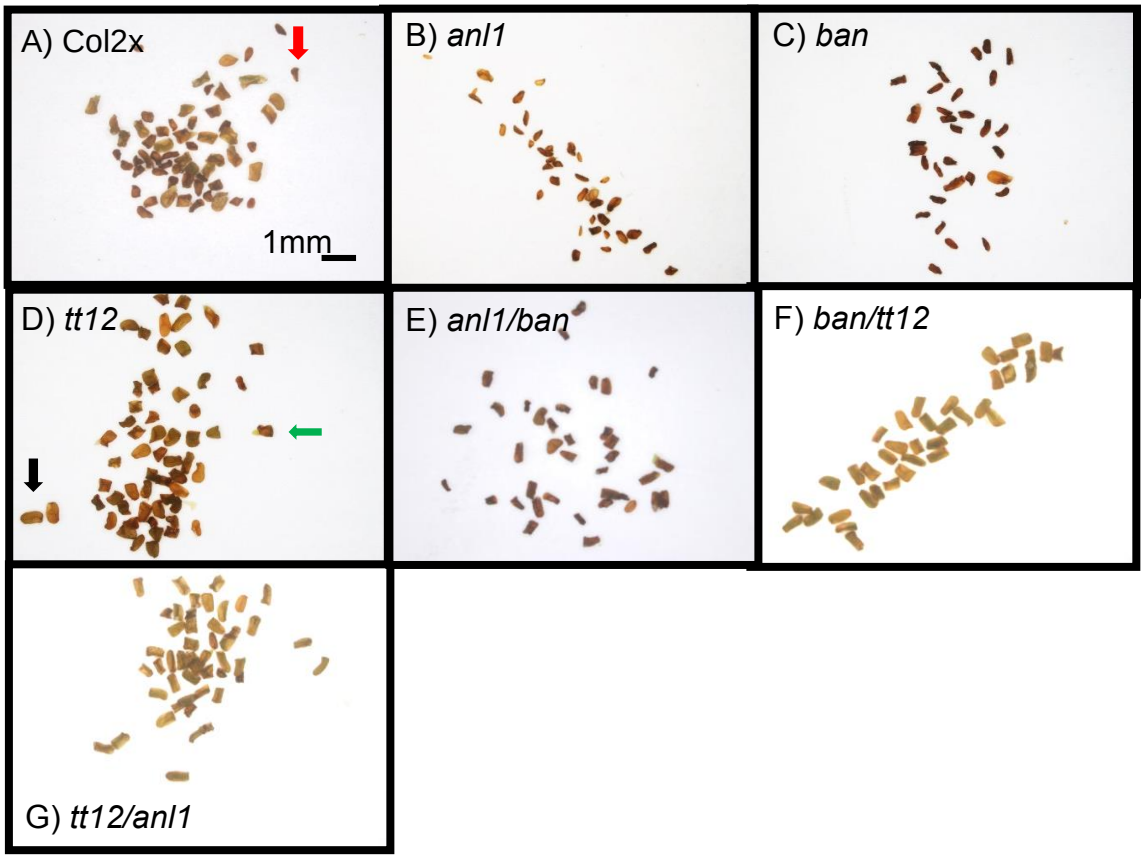


Figure 4.7: Morphological analysis of mature seeds derived from crosses between FBP double mutants and Col4x pollen parents. Differentiating morphology and size of mature seeds resulted in classifying seeds into two classes based on their potential viability: a) 'plump and burst' seeds together represent the 'rescue' category of seed and b) the 'shrivelled' category represents likely unviable seed. (**A**) Seeds from the control cross Col2x X Col4x. (**B**) to (**G**) seeds derived from single and double FBP mutants crossed with Col4x pollen parents. All the images of mature dry seeds were taken at the same light intensity and magnification, to make clear comparisons of seed shape, size and colour. Seeds were classified and counted, as 'plump' 'burst' and 'shrivelled' and examples are black, green and red arrows respectively. Scale bar = 1mm.

The percentage of 'plump and burst' seeds was then calculated for each silique and mean percentages for the 'plump and burst' class were plotted (Figure 4.8). The highest proportion of 'plump and burst' seeds resulting from crosses to Col4x was for *tt12*, with a mean of 54%. Following that, the proportion of 'plump and burst' seeds that were significantly different from the control cross were derived from *ban/tt12*, *ban* and *tt12/anl1*, with means of 27%, 18% and 17% respectively. However, Col2x, *anl1* and *anl1/ban* crossed to Col4x had the lowest proportion of plump and burst seeds, with means of 6%, 5% and 4% respectively (Figure 4.8). A 'rescue ratio' was calculated for the progeny of each cross for ease of comparison to seeds from the Col2x X Col4x control (see table 4.5 in Appendix B).

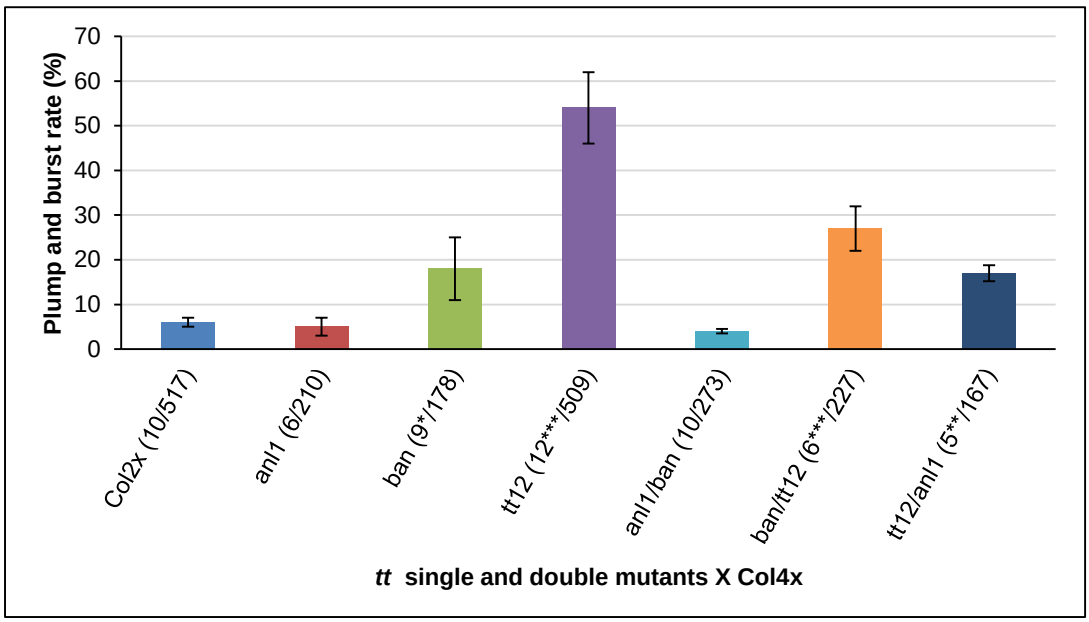


Figure 4.8: Mean % of 'plump and burst' seeds derived from crosses between maternal FBP double mutants and Col4x pollen parents. 'Plump and burst' seeds were considered as likely viable mature seeds. Error bars represent standard error. Numbers in brackets represent a number of siliques used and seed numbers. Significant differences were shown by a Mann-Whitney test: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$.

4.2.4 Summary of FBP single and double mutants data

All data collected relating to the stage of embryo development, cellularisation of endosperm, areas of the maximum cross-section of the embryo sac, chalazal endosperm areas and number of nodules, mature seed weight, classification of mature seed phenotype and seed viability for crosses involving FBP single and double mutants crossed to Col4x has been summarised in Table 4.6. In this chapter, a broad range of data has been collected in order to assess whether the use of double mutants of the FBP can be informative in clarifying the role of particular pathway intermediates and end products in the phenomenon of Col4x-induced seed lethality. The meaning of this data will be discussed in the next section.

Table 4.6: Summary of all seed data collected for progeny derived from FBP single and double mutants (and their respective progenitor lines) crossed with Col4x pollen parents. All plant lines detailed in column 1 represent the maternal partner used in crosses to Col4x pollen parents. The weight of mature seeds was considered a reasonable measure of seed 'rescue' from Col4x induced lethality and thus, seed weights of seeds resulting of FBP mutants crossed to Co4x are organised in descending order. Values are described as the mean ± S.E. Sample sizes (**n**) are in brackets. In columns 2 and 3 (embryo stage), black-shaded cells represent an observation of cellularisation of the peripheral endosperm**. Abbreviations: nd**: no data; ◆: no rescue ratio value obtained.

Genotype	**Seed development at 5 and 7DAP**								**Mature seeds**			
	Embryo stage		**Embryo sac area (µm ²)**		**Chalazal endosperm area (µm ²)**		**Number of nodules**		**Seed weight (µg)**	**% 'Plump & burst' seed**	**Weight ratio**	**'Plump and burst' ratio**
	5 DAP	**7 DAP**	**5 DAP**	**7 DAP**	**5 DAP**	**7 DAP**	**5 DAP**	**7 DAP**				
Col2x X Col2x (reference cross)	heart	torpedo	nd	nd	nd	nd	0	0	17± 3 (5)	◆	◆	◆
Col2x	early heart	overgrown globular retarded	40026 (10)	50771 (10)	2645 (10)	6312 (10)	0.8 (10)	0.5 (10)	14 ± 2 (517)	6 ± 1	◆	◆
ban/tt12	globular	Heart	43100 (10)	119737 (10)	1875 (10)	12119 (10)	1.7 (10)	0.7 (10)	25± 7 (227)	27 ±5	1.8	4.5
tt12	heart	overgrown globular retarded	43772 (10)	47249 (10)	2069 (10)	4274 (10)	1.3 (10)	1.2 (10)	21 ± 1 (509)	54 ± 8	1.5	9
tt12/anl1	globular	abnormal overgrown globular	33054 (10)	81171 (10)	1526 (10)	4232 (10)	0.8 (10)	1.1 (10)	18± 3 (167)	17 ±1.8	1.3	2.8
anl1/ban	globular	abnormal overgrown globular	47056 (10)	77185 (10)	1046 (10)	5343 (10)	1.3 (10)	3.7 (10)	16± 2 (273)	4 ±0.5	1.1	0.7
ban	heart	heart overgrown retarded	40540 (10)	101176 (10)	881 (10)	6430 (10)	1.9 (10)	2.4 (10)	14 ± 1 (178)	18 ± 7	1	3
anl1	globular	globular	40931 (10)	80717 (10)	764 (10)	52555 (10)	0.7 (10)	1.9 (10)	6 ± 1 (210)	5 ±2	0.4	0.8

4.3 Discussion

From the data presented in chapter 3, several seed lines resulting from crosses between FBP single mutants and Col4x indicated that perturbation of flavonoid biosynthesis could lead to the partial rescue of Col4x-induced seed lethality. This work highlighted the importance of the flavonoids generated after the cyanidin stage of the pathway and thus mutants *anl1, ban,* and *tt12* were chosen for further functional genomic analyses in combinations as double mutants. As stated in the introductory section (see Chapter1: section 1.6.2.6), the above-mentioned mutants have an important role in the synthesis and transport of anthocyanin and proanthocyanidin, which are two flavonoids responsible – among other functions - for giving the seed coat its distinctive colour (Debeaujon *et al.,* 2003). Moreover, there is currently not enough information on the roles of anthocyanin and proanthocyanidin in seed viability, though seed pigmentation has been linked with seed germination (Mavi, 2010; Ertekin and Kirdar, 2010).

The impact of these mutants was further studied by dissecting siliques and analysing the seed morphology and seed coat colour. Results showed that seeds derived from *tt12/anl1* and *ban/tt12* crosses to Col4x have a similar seed shape and relatively similar seed coat colour. The seed coat colours observed could be due to the accumulation of flavonols (yellow) (Pourcel *et al.,* 2005), and to the high levels of cyanidin (pink to red) (Winkel-Shirley, 2001), that would likely accumulate following mutation in the synthesis of anthocyanin and proanthocyanidin. It is noteworthy at this point to state that anthocyanin and proanthocyanidin would normally be responsible for giving the seed coat its colour once imported into the vacuole; thus, if *TT12* is not functional, this would lead to a problem in the transport to the vacuole, and hence a change in the seed coat colour. Additionally, in *tt12* mutants, *TT19* would aid anthocyanin transport to the vacuolar membrane, hence, allowing some of the anthocyanins in the vacuole via other tonoplast localised transporters (Sun *et al.*, 2012; Shi and Xie, 2014).

Analysis of the developing seeds by confocal laser microscopy showed that only *ban/tt12* had a “delayed” heart stage, which is an indication that the seed could be viable. Indeed many mature seeds derived from crosses to this double mutant were plump and thus, *ban/tt12* can be considered to rescue Col4x-induced lethality. This finding is consistent with reports about delayed seed development of seeds originating from paternal Col4x and maternal FBP mutations (Dilkes *et al.,* 2008). The fact that no change from the globular stage was apparent in seeds derived from *tt12/anl1* and *anl1/ban* lines do not

mean that these seeds would not be viable; rather, it could be that their development is delayed substantially, and that they might show a transformation in stages with time (e.g. 9DAP) (Dilkes *et al.*, 2008). The data from mature seed phenotypes suggest that *tt12/anl1* plants do rescue Col4x-induced lethality but that the progeny of *anl1/ban* seed parents were mostly inviable. Given the data obtained in this work a future study should examine embryo stages at 9 and 12 DAP to clarify how development proceeds in these crosses.

Further analysis of the development of the seeds resulting from crosses with maternal FBP mutants was performed by measuring embryo sac area, chalazal endosperm area, and the number of nodules, as some studies has suggested a link between these parameters and seed viability (Johnson *et al.*, 2002; Garcia *et al.*, 2003; Garcia *et al.*, 2005; Dilkes *et al.*, 2008).

Here these parameters were found show some trends relating to a rescue but overall to be fairly unreliable indicators of 'rescue' for FBP mutants in the Col-0 ecotype. This could be due to the fact that overall the ability of FBP mutants to overcome Col4x-induced seed lethality in the Col-o ecotype is poor compared to other ecotypes examined to date and seed development aberrant in all crosses, including those that are considered 'rescue' phenotypes. Trends in the data are discussed in the next section.

4.3.1 Interpretation of data from seed development stages

Average embryo sac area was significantly increased at 7 DAP in all seeds resulting from maternal FBP mutants, except for seeds arising from *tt12*. The *tt12* single mutant was a reliable rescuer of Col4x-induced seed lethality and thus, restriction of endosperm proliferation might be the cause of this. Measurements taken at 5 DAP showed no significant difference between the control/diploid and the mutant polyploidy seeds. Comparison between seeds resulting from single FBP mutants and double FBP mutants might lead to postulate that anthocyanin might have a central role in the enlargement of the embryo sac area. This postulation is based on the observation that:

a) When proanthocyanidin synthesis is blocked in *ban*, this could lead to more synthesis of anthocyanin due to the potential over-accumulation of cyanidin; increased levels of anthocyanin may thus be responsible for the larger embryo sac observed in crosses involving this mutant - anthocyanin would be transported to the vacuole but much may remain in the cytosol. *ban/tt12* would block import of anthocyanin to the vacuole and thus even higher levels of anthocyanin could

accumulate in the cytosol, hence, the size of the embryo sac would grow even larger;

b) Seeds resulting from *anl1* single and double mutants (*anl1/ban* and *tt12/ anl1*) produced embryo sacs of the similar area (although enlarged compared to control and smaller than those for *ban* and *ban/tt12*). This could suggest that the role of accumulation of proanthocyanidin in embryo sac enlargement is limited, and in fact, the bigger role is for anthocyanin.

Results from measurements of the chalazal endosperm did not appear to show convincing trends as was found for embryo sac area. At 5 DAP, only seeds with maternal *anl1*, *ban*, and *anl1/ban* mutants showed a reduced chalazal endosperm area. However, results were inconclusive when compared to each other and to the control. It is suggested to repeat this experiment with higher numbers of replicates, and to include more mutants from the FBP, such as *tt19* and other enzymes working upstream and downstream of anthocyanin and proanthocyanidin. At 7DAP, only seeds derived from *ban/tt12* had significantly larger chalazal endosperm areas. Again, results were not conclusive.

Similarly analysis of the number of nodules numbers was not instructive. Again, it is suggested that this experiment could be repeated with a larger sample size coupled to use of more FBP mutants.

4.3.2 Interpretation of results from mature seeds

Comparison of mature seeds derived from self-pollination FBP double mutants and those resulting from crosses to Col4x pollen parents further confirmed that the shrivelled abortive seed phenotype is caused by parental Col4x (Figures 4.1 and 4.7).

Mature seed weight is an indicator of embryo weight, and thus could provide some insight into seed viability (Schruff *et al.*, 2006). A definite trend in the seed weight data showed that seeds derived from seed parents carrying the *tt12* mutation (*tt12*, *ban/tt12*, and *tt12/ anl1*), had higher seed weights than the other lines investigated. Thus, TT12 appears to be a central player in the regulation of seed development but it is unclear whether this is via altering the flux and accumulation of various FBP intermediates or through influencing vacuolar maturation (*tt12* mutants have abnormal vacuoles in the endothelium) (Baxter *et al.*, 2005; Kitamura *et al.*, 2010; Shi and Xie, 2014). It is also noteworthy that seeds derived from *tt12/anl1* had lower seed weight, which is probably linked to the presence of *anl1*, which as a single mutant produced a high percentage of

embryo lethal seeds in crosses to Col4x. It is tempting to speculate that this could be due to the fact that more FBP product is pushed towards proanthocyanidin accumulation in the vacuole however *ban* mutants (which lack proanthocyanidins) (Devic *et al.*, 1999; Abrahams *et al.*, 2002; Kubo *et al.*, 2007; Marinova *et al.*, 2007) fail to rescue Col4x-induced seed lethality.

Finally, the percentage of plump and burst seed was calculated, as this appears to provide a reliable measure of seed 'rescue' from Col4x-induced lethality (Dilkes *et al.*, 2008). Again, seeds with maternal *tt12* in their genome (*tt12*, *ban/tt12*, and *tt12/anl1*) had a higher percentage of "rescue" seeds, indicating the importance of TT12 in interploidy lethality (Figure 4. 8). However, in *ban/tt12* X Col4x and *tt12/anl1* X Col4x crosses the impact of *ban*, and *anl1* mutants respectively were stronger than *tt12*. Whereas in *anl1/ban* X Col4x cross the effect of *anl1* mutant is stronger than *ban* (Figure 4. 8).

In conclusion, analysis of the mature seed data highlighted that functional TT12 plays a negative role in the rescue of Col4x-induced seed lethality, and that disruption its function leads to increased levels of anthocyanin and proanthocyanidin precursor (or their downstream targets) in the cytoplasm (Petrussa *et al.*, 2013). Moreover, due to the rescue phenotypes associated with *ban/tt12*, it is suggested that cytoplasmic anthocyanin plays a decisive role during seed development such that seeds avoid the post-zygotic hybridisation barrier. Alternatively, in cannot be ruled out that mutation of the genes utilised in this study affect overall regulation of the pathway in a manner that is difficult to predict. If this were the case, then the concentrations of products and intermediates that are produced earlier in the pathway (such as flavonols) could be significantly affected and impact on seed development.

4.4 Limitations

This part of the project had limitations that contributed to a degree of uncertainty in the data sets and thus the conclusions that could be drawn from them. In particular, the relatively small sample size for some of the double mutant data impacted on statistical significance. Also, it proved impossible to recover double mutants for *fls* and *anl1* - this would have permitted further consideration of the potential role of flavonols in modulating seed development as well as further increasing flux through the FBP to proanthocyanidins in this particular double mutant combination. The failure to recover this double mutant suggests that the double homozygous seeds were non-viable.

4.5 Future perspectives

Several aspects of the FBP would benefit from the closer study. In particular, the generation of more combinations of FBP double mutants (e.g. *tt3* with *fls*) and triple mutants (e.g. *anl1; ban; tt12*) in different positions in the pathway would be potentially informative to dissect the links between flavonoid metabolites and endosperm cellularisation.

Chapter 5 Quantification of different flavonoid subclasses in wild-type and *tt* mutant seed at the developmentally important heart stage

5.1 Introduction

Flavonoids are present in most plant parts in a free form; for example, flavonoid glycosides, which combine one or two glucoside and/or rhamnoside residues that are esterified in the third and/or seventh position, are found in vacuoles of stems, roots, leaves and flowers, while flavonoid aglycones (kaempferol and isorhamnetin) are found in wax on bark, leaves and buds or as crystals in the cells of cacti (Di Carlo *et al.*, 1999; Lepiniec *et al.*, 2006).

5.1.1 The classification of flavonoids in seeds

As mentioned in the introduction of Chapter 3, the main types of flavonoids in seeds are flavonols, anthocyanins, proanthocyanidins (PAs) and isoflavones, (Lepiniec *et al.*, 2006). Lepiniec and colleagues (2006) have suggested that flavonols and PAs only accumulate in the mature seeds of *A. thaliana* mature and constitute, about 50% of the measurable flavonoids. The biosynthesis of PAs begins early in seed development, first appearing in the endothelium layer of the integuments at the micropylar end of the nascent seed coat at around 1 to 2 DAP; accumulation continues in the endothelium toward the chalaza until about 5 to 6 DAP (Shirley, 1998).

Glycoside derivatives are also considered very common flavonol forms (Lepiniec *et al.*, 2006), residing in the embryo and the testa in mature seeds (Routaboul *et al.*, 2006). Lepiniec *et al.*, (2006) reported the presence of other aglycones (kaempferol and isorhamnetin), as well as glycosides that combine one or two rhamnoside and/or glucoside residues esterified in positions 3 and/or 7. However, the main flavonol is quercetin (also called quercetin-3-O-rhamnoside).

5.1.2 Analytical methods for the detection and quantitation of flavonoids

Mass Spectrometry (MS) is the most efficient method for biomedical analysis, in particular when biological samples consisting of complex biochemical mixtures require analysis. The primary benefits of MS are its high level of sensitivity enabling analysis of

substances in the μg range, and high specificity, which allows resolution between molecules of the same molecular weight but different atomic composition, and even to occasionally differentiate stereoisomers. MS advantage also easily couples to other separation methods such as High-Performance Liquid Chromatography (HPLC) and gas chromatography to allow greater sensitivity and discrimination where necessary. MS has been used extensively to analyse flavonols, anthocyanins and isoflavones (Cuyckens and Claeys, 2004; Valls *et al.*, 2009; Pinheiro and Justino, 2012.).

5.1.3. Aims and objectives

The purpose of the work described in this chapter was to identify the seed flavonoids responsible for the ability of certain FBP mutants to mitigate the Col-0 killer seed lethality effect. The first experimental objective was to use liquid chromatography– mass spectrometry (LC-MS) to perform a comprehensive characterisation of the flavonoids of *A. thaliana* FBP mutants and wild-type seeds in both the Col-0 and L*er* ecotypes. The 5DAP time point in seed development was chosen for two reasons: a) the accumulation of flavonoids in the endothelium peaks, potentially leading to a maximum impact on seed development, and b) the Col-0 killer effect is strongly associated with the failure of seed development to progress beyond heart stage (5DAP in our growth conditions); this failure is in turn strongly correlated with late or absent endosperm cellularisation (see Chapter 3). Individual FBP mutants allow progression beyond this point by promoting cellularisation of the endosperm (see Chapter 3). Hence, it was reasoned that comparing the flavonoid profiles of the key FBP mutants (i.e. Col-0 killer rescuing (*tt4* and *ttg2*) and non-rescuing (*fls*, *anl1*, *ban*)) might reveal the flavonoid(s) responsible for the relieving the Col-0 killer effect.

A further objective was to measure the levels of auxin in the various FBP mutants at 5DAP. This was attractive given the documented ability of flavonoids to interfere with the polar transport of this important growth regulator and the possibility therefore that the FBP regulates endosperm development via auxin (see Chapter 6).

5.2 Results

5.2.1 The accumulation of various flavonoids in 5DAP wild-type seed of the *A. thaliana* ecotypes Col-0 and L*er* ecotypes

The profiles of accumulated flavonoids in 5DAP seed of mutant and wild-type plants were analysed by liquid chromatography–electrospray ionization triple quadruple mass spectrometry (LC-ESI- QQQ-MS/MS). The MS studies identified metabolites through a total analysis of the patterns of mass fragmentation and comparison to known compounds, as listed in Chapter 2 - Table 2.1 and Table 2.2. Data from flavonols, soluble procyanidin and anthocyanin molecule 1 (A1) in the Col-0 and L*er* ecotypes was summed and normalized to an internal standard (umbelliferone).

Flavonols, soluble procyanidin polymers and anthocyanin molecule 1 (A1) were detected. The most abundant flavonol detected in wild-type Col-0 and L*er* ecotypes was the quercetin-rhamnoside-hexoside (Q-R-H) with normalized peak areas of 34.49 (Col-0) and 36.45 (L*er*) (Figures 5.1.A and 5.2.A). In both ecotypes, the derivative profile of quercetin corresponded to a) quercetin-3-O-rhamnoside, displaying different peak area and retention time (9.8 minutes (m), 10.8m and 11.6m), b) quercetin-di-rhamnoside, c) quercetin-hexoside-rhamnoside and d) quercetin-3-O-glucoside.

In both ecotypes, there were relatively low, but detectable levels of kaempferol at the retention times 10.1 m, 10.75 m and 12.1 m. There was also a record of minor products of kaempferol derivatives: a) kaempferol-rhamnoside at the retention times of 10.1m, 10.4m and 10.6m; b) kaempferol-3,7-di-O -rhamnoside and, c) kaempferol-rhamnoside-hexoside. The characteristics of kaempferol-3-O-glucoside-7-O-rhamnoside and kaempferol-3,7-di-O-rhamnoside are presented in Table 2.2. Isorhamnetin-rhamnoside, isorhamnetin-di-rhamnoside and isorhamnetin-hexoside-rhamnoside are isorhamnetin derivatives that form flavonol glycosides (Table 2.2).

Soluble procyanidin appeared as a multimer: dimer, trimer, tetramer, pentamer and hexamer (Table 2.2; Figure 5.1.B). In both Col-0 and L*er* ecotypes, the peak areas of trimer procyanidin were the highest, followed by tetramer, pentamer, dimer and then hexamer procyanidin.

Finally, profiles showed that the levels of A1 were 17.6% higher in Col-0 compared to L*er* ecotype (Figure 5.3).

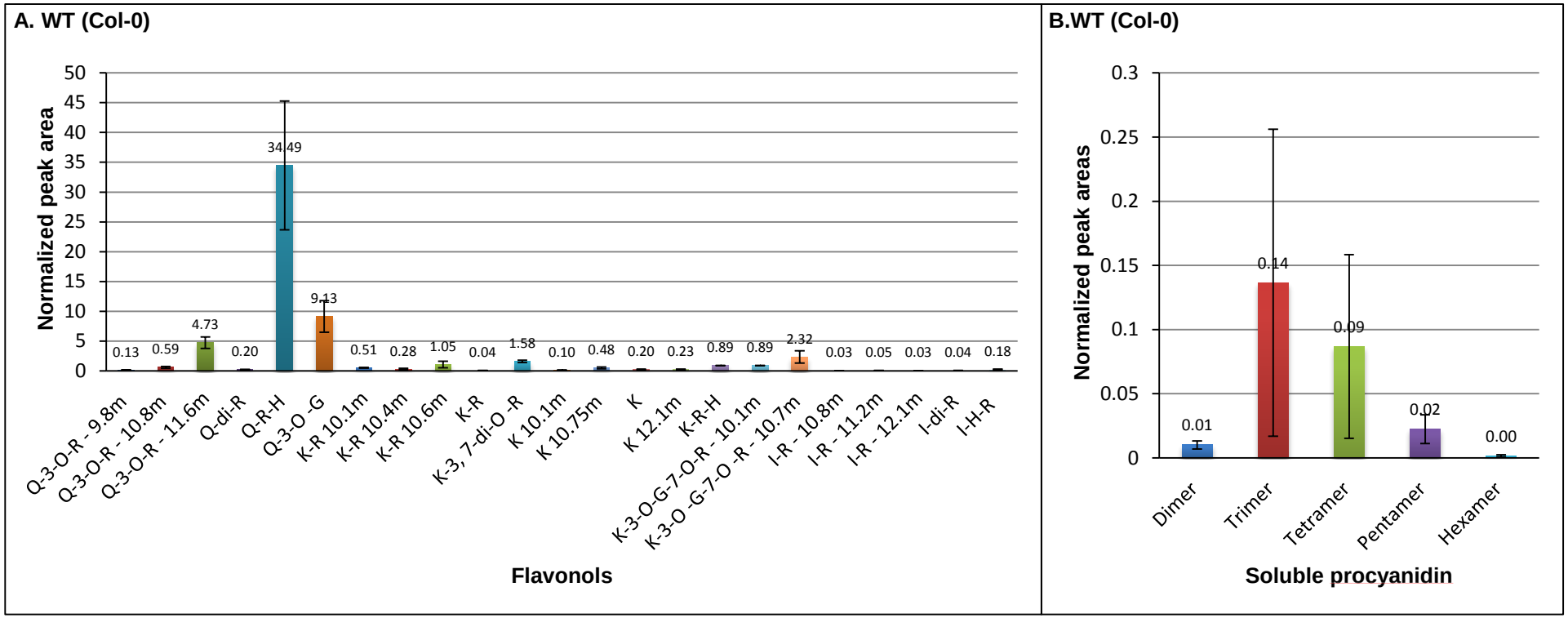


Figure 5.1. LC-ESI- QQQ-MS/MS derived flavonoid profiles in 5 DAP seeds of *A. thaliana*, Col-0 ecotype. A. Flavonol content. **B.** Soluble procyanidin content. Mean values ± standard error of three independent measurements. Data from flavonols and soluble procyanidin in the Col-0 and L*er* ecotypes have been summed and normalized to an internal standard (umbelliferone). **Abb. G:** glucoside, **H:** hexoside, **I:** isorhamnetin, **K:** kaempferol, **m:** retention time, **Q:** quercetin, **R:** rhamnoside.

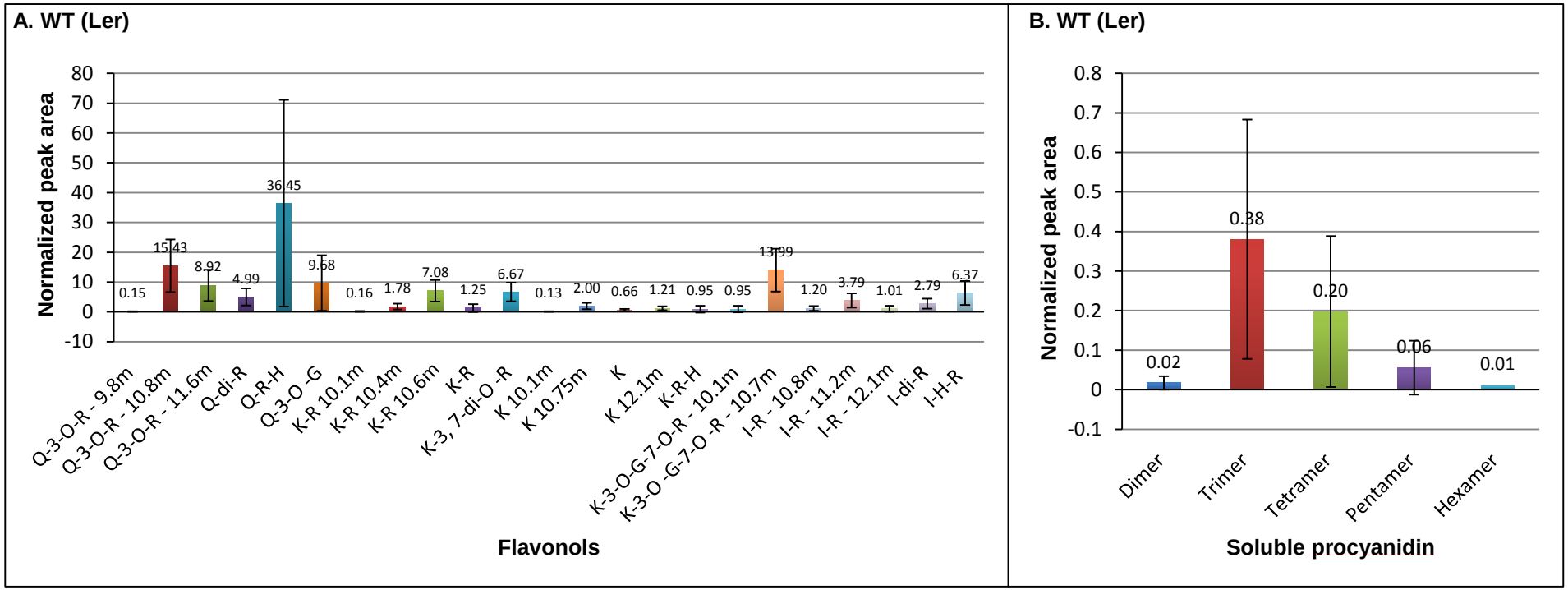


Figure 5.2: A list of LC-ESI- QQQ-MS/MS results showing flavonoid composition in 5 DAP seeds of *A. thaliana*, L*er* ecotype. A. Flavonol contents. **B.** Soluble procyanidin. Mean values ± standard error of three independent measurements. Data from flavonols and soluble procyanidin in the Col-0 and L*er* ecotypes have been summed and normalized to an internal standard (umbelliferone). **Abb. G:** glucoside, **H:** hexoside, **I:** isorhamnetin, **K:** kaempferol, **m:** retention time, **Q:** quercetin, **R:** rhamnoside.

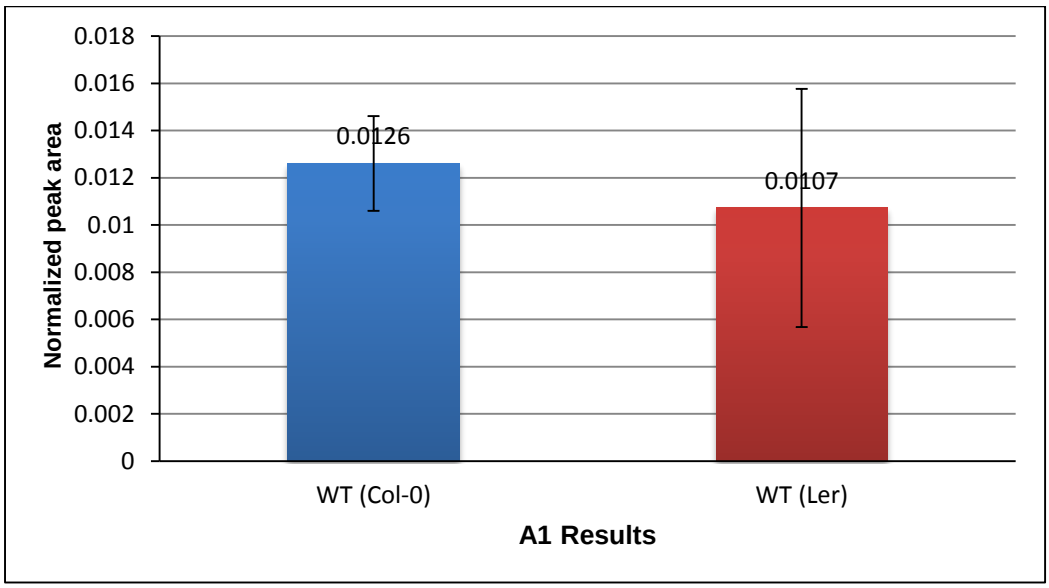


Figure 5. 3: LC-ESI- QQQ-MS/MS results showing levels of anthocyanin molecule 1 (A1) in 5 DAP seeds of *A. thaliana*, Col-0 and L*er* ecotypes. Data from A1 in the Col-0 and L*er* ecotypes have been summed and normalized to the internal standard umbelliferone.

5.2.2 The analysis of flavonoids in FBP mutants and their A. thaliana ecotypes

LC-ESI- QQQ-MS/MS revealed that 5DAP seed of *tt4* in both ecotypes contained very low levels of detectable flavonols compared to their respective wild-type. In Col-0, levels were reduced to 20% of the wild-type value, whilst in the L*er* the impact was more dramatic with levels near the limit of detection i.e. close to a 100% reduction (Figure 5.4). The levels of flavonols were also much lower in the *ttg2* mutant in L*er* compared to its wild-type, but flavonol levels for the same mutant in Col-0 were similar to its wild-type. Interestingly, flavonol levels in Col-0 wild-type and the three non-rescuing FBP mutants, *fls*, *anl1*, and *ban* were similar.

Soluble procyanidins were undetectable in *tt4* in both ecotypes, and in *ttg2* in the Col-0 background; however, extremely low levels of the pentamer derivatives were detected in *ttg2* in the L*er* background (Figure 5.5). It is also noteworthy that the levels of trimer and tetramer procyanidin derivative were substantially higher in *fls* and *ban* compared to their Col-0 control. In contrast, *anl1* had similar levels of procyanidin to Col-0 control (Figure 5.5).

A1 levels were consistently very low in *tt4* and *ttg2* in both ecotypes. Additionally, *anl1* displayed, on average, lower levels of A1 than Col-0 control (Figure 5.6).

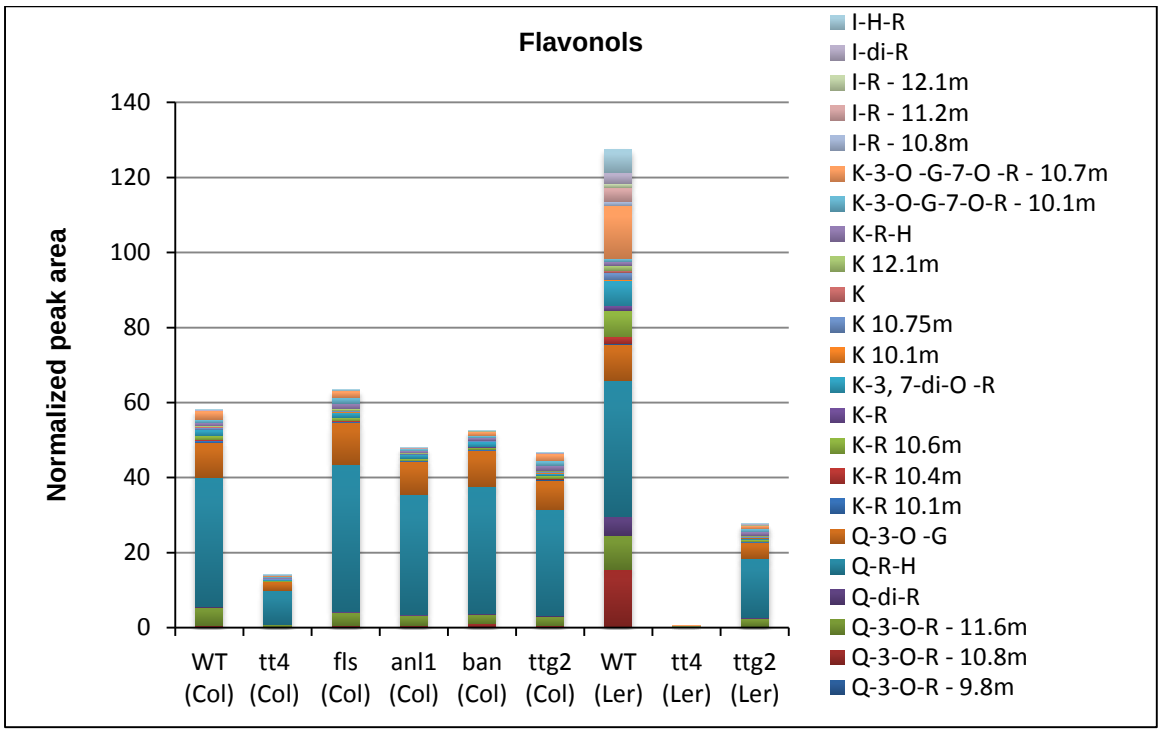


Figure 5.4: Quantification of flavonols in FBP mutants in *A. thaliana* Col-0 and L*er* ecotypes. Data from flavonols in the Col-0 and L*er* ecotypes have been summed and normalized to an internal standard (umbelliferone) using (LC-ESI- QQQ-MS/MS) analysis of three independent measurements. Results showed a significant difference between *tt* mutants in both ecotypes by using Mann-Whitney test ($P \leq 0.05$; data not shown here). **Abb. G:** glucoside**, H:** hexoside, **I:** isorhamnetin, **K:** kaempferol, **m:** retention time, **Q:** quercetin, **R:** rhamnoside.

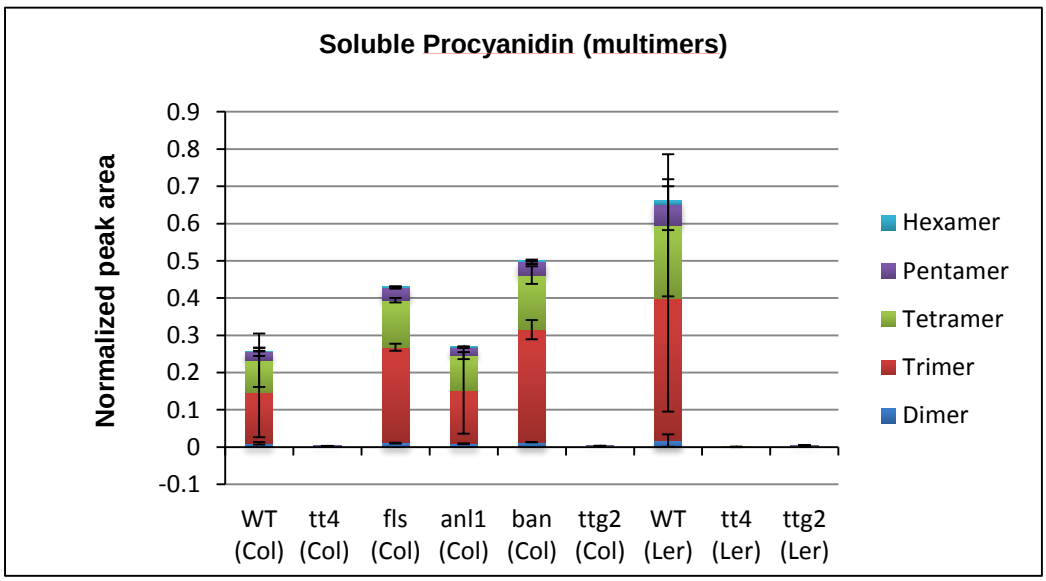


Figure 5.5: Quantification of soluble procyanidin in FBP mutants in *A. thaliana* Col-0 and L*er* ecotypes. Data from soluble procyanidin in the Col-0 and L*er* ecotypes have been summed and normalized to an internal standard (umbelliferone), measured using (LC-ESI- QQQ-MS/MS) analysis. The soluble procyanidin results were tested for significant difference between mutants and their controls using Mann-Whitney Test. Procyanidin trimer, tetramer, and pentamer levels were significant – $P \leq 0.05$- different between *tt* mutants and their controls in both ecotypes (data not shown here). **Abb. G:** glucoside, **H:** hexoside**, I:** isorhamnetin, **K:** kaempferol, **m:** retention time, **Q:** quercetin, **R:** rhamnoside.

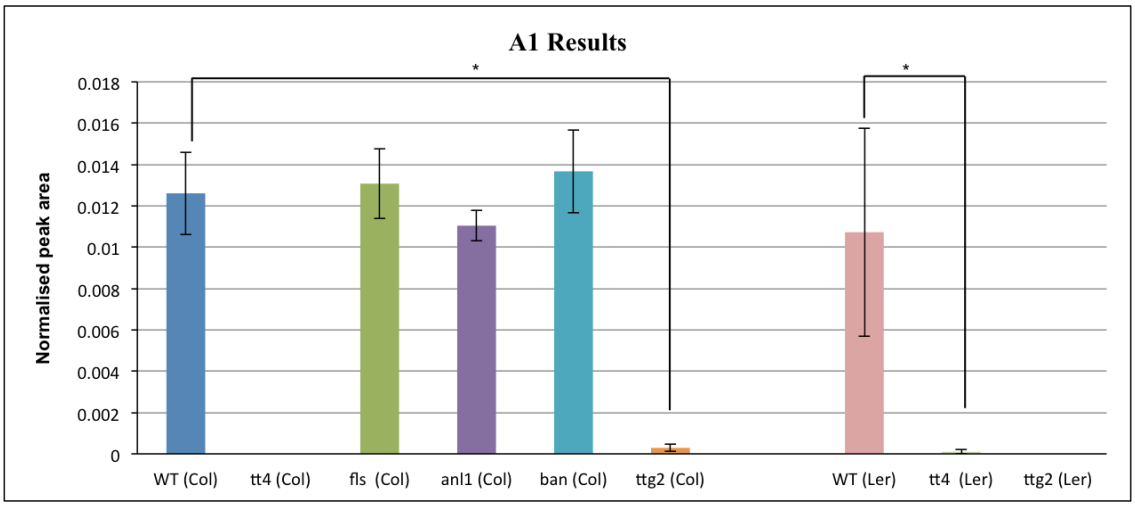


Figure 5.6: Quantification of A1 in FBP mutants in *A. thaliana* Col-0 and L*er* ecotypes. Data from A1 in the Col-0 and L*er* ecotypes have been summed and normalized to an internal standard (umbelliferone) and measured using (LC-ESI-QQQ-MS/MS) analysis of three independent measurements. Bars labelled * were shown *tt* mutants that were statistically significantly different from the control data $P \leq 0.05$.

5.3 Discussion

The work reported in this chapter aimed to analyse the change in the level of flavonols and flavonoids resulting from mutations in genes in the FBP in *A. thaliana* associated with rescue or non-rescue of the Col-0 killer effect. Quercetin, kaempferol, and their glycosides were chosen as representatives for flavonols, and anthocyanin 1 (A1) and soluble procyanidin were chosen as representatives for anthocyanins and proanthocyanidins, respectively (He *et al.*, 2008; MacGregor *et al.*, 2014). A further consideration in this choice was that these classes of flavonoids have different temporal synthesis profiles: flavonols are synthesised midway along the pathway, whilst A1 and procyanidins are synthesised towards its terminus (Figure 5.7).

The most significant motivation however came from the evidence that flavonols block transport of auxin (Jacobs and Rubery, 1988; Peer *et al.*, 2001; Peer *et al.*, 2004; Besseau *et al.*, 2007; Santelia *et al.*, 2008; Buer *et al.*, 2013), a plant growth regulator which may play an important role in regulating endosperm cellularisation (Lur and Setter, 1993) a proposal given significant support by recently published data (Figueiredo *et al.*, 2015). It was expected therefore that if a causal link exists between auxin transport, flavonol accumulation and the behaviour of the endosperm in interploidy crosses, the pattern of accumulation or depletion of flavonols in the various FBP mutants compared to wild-type should correlate with their impact (positive or negative) on the Col-0 killer effect.

Anthocyanins were chosen for further analysis based on observations from Chapter 4 that they play a role in the determining embryo sac area, which like the timing of endosperm cellularisation, is a factor in seed rescue in interploidy crosses (see Chapter 4 section 4.3). Proanthocyanidins were chosen for analysis due to their role in the oxidative browning of seeds -dark brown seed colour is usually correlated with lethality in the Col-0 -killer effect - in addition to playing a role as the counterparts of anthocyanins following synthesis from cyanidin (see Chapter 4 section 4.3.1 for comparison between *anl1* and *ban*).

The results of LC-ESI-QQQ-MS/MS in both Col-0 and L*er* ecotypes showed that flavonol levels were much higher than procyanidin and A1 levels. This could be due to the corresponding position of the flavonols in the FBP but also, and more importantly, due to their relative importance in plant functions (Emiliani *et al.*, 2013). Moreover, levels of soluble procyanidin were higher than levels of A1, which could be due to the higher variability in the subclasses of proanthocyanidins, as compared to anthocyanins.
The data revealed that flavonol levels were much higher in L*er* than in Col-0. In fact, the difference correlates with higher levels of the precursors, Q-3-O-R, kaempferol, and flavonol glycosides (Figure 5.4). This observation is interesting given the ability of a wild-type L*er* seed parent to substantially rescue the viability of seed resulting from a 2xXCol4x cross (see Chapter3: sections 3.2.2.1 and 3.2.2.2; Dilkes *et al.*, 2008) and suggests that high levels of flavonols in the endothelium at 5DAP are associated improved endosperm cellularisation, since this is the most significant determinant of seed viability in these interploidy crosses.

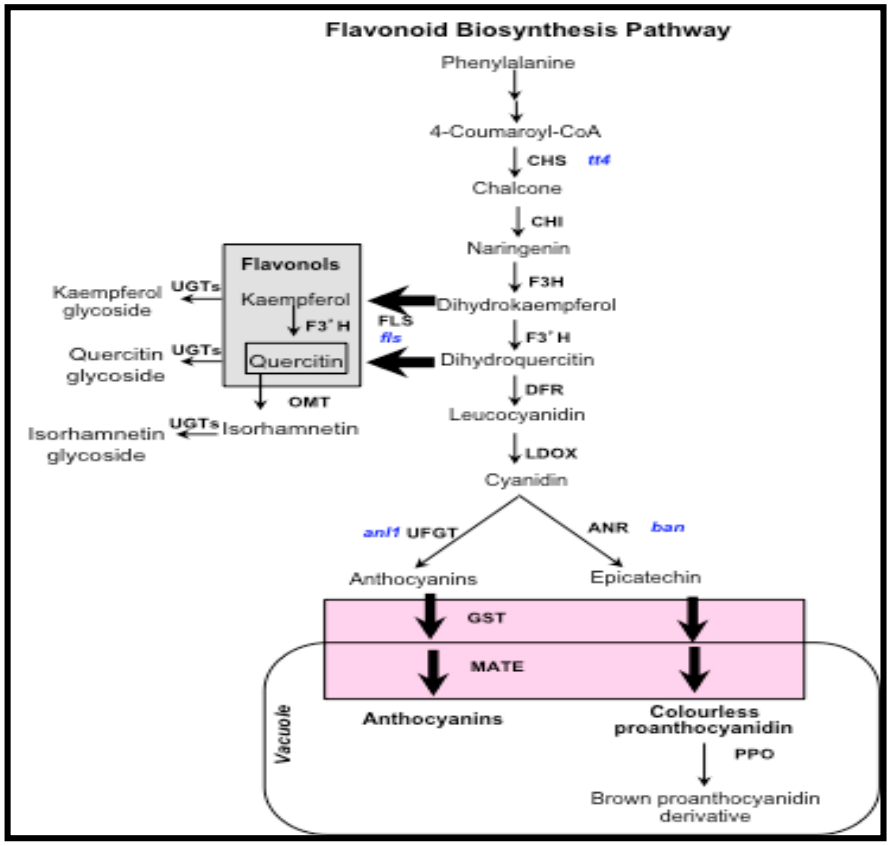


Figure 5.7: The FBP pathway in *A. thaliana* seeds. The diagram shows the position of the various FBP mutants analysed by LC-ESI-QQQ-MS/MS in this study. *TTG2* is not displayed, but is a transcription factor that regulates PAs biosynthesis (Haughn and Chaudhury, 2005). Enzymes are shown with a black colour and their names are: **CHS:** Chalcone synthase; **CHI:** Chalcone isomerase**; F3H:** Flavanone-3-hydroxylase**; F3'H:** Flavonoid-3'-hydroxylase**; FLS:** Flavonol synthase**; DFR:** Dihydroflavonol 4-reductase**; LDOX:** Leucoanthocyanidin dioxygenase**; UGFT:** UDP-glucose: flavonoid 3-O-glucosyltransferase**; ANR:** Anthocyanidin reductase**; GST:** Glutathione S-transferase**; MATE:** MATE secondary transporter; **AHA10:** Autoinhibited H^+-ATPase 10; **PPO:** Polyphenol oxidase; **UGTs:** UDP-dependent glycosyltransferase and **OMT:** *O*-methyltransferase (Modified from Winkel-Shirley, 2001; Peer *et al.*, 2001; Debeaujon *et al.*, 2003; Baxter *et al.*, 2005; Pourcel *et al.*, 2005; Lepiniec *et al.*, 2006; Peer and Murphy, 2007; Kleindt *et al.*, 2010; Gou *et al.*, 2011; Appelhagen *et al.*, 2014; Ishihara *et al.*, 2016).

Although not attempted in the present work it would be informative to determine the effect(s) of the gain of function, rather than the loss of functions mutations tested in Chapters 3 and 4 of some genes like *FLS*, *ANL1*, and *BAN*, using 35S promoter transcriptional fusions for example. The same argument could be made about the levels of soluble procyanidin. Assuming a positive relationship exists between high flavonol levels and seed rescue, such over-expression should further improve seed viability in 2xX Col4x crosses.

How does this hypothesis fit with the data from the FBP mutants? As expected, at 5DAP both *tt4* and *ttg2* had lower levels of flavonols, soluble procyanidin, and A1 in both Col-0 and L*er*. The impact of *tt4* on flavonol levels is explained by the fact that *TT4* encodes

the first committed enzyme, chalcone synthase (CHS), of FBP and therefore, in agreement with the analysis of mature seeds Routaboul *et al.* (2006), no flavonoids were expected in *tt4* seeds (Figure 5.8). The effects in *ttg2*, however, are more complicated (Garcia *et al.*, 2005). Evidence suggests that TTG2 is involved in the biosynthesis of proanthocyanidin in the later stages of the pathway (Debeaujon *et al.*, 2003; Baxter *et al.*, 2005). This was consistent with analysis of 5DAP seeds in *ttg2* mutants of both Col-0 and L*er*, which showed almost complete absence of proanthocyanidin (Figure 5.5). However, the results presented in Figures 5.4 and 5.6 suggest a potentially wider role for this transcription factor, since *ttg2* mutant seed in the L*er* ecotype also showed a very substantial reduction in total flavonol levels relative to the wild-type (Figure 5.4). However, the same mutation in the Col-0 ecotype had almost wild-type levels of total flavonols. Anthocyanin A1 levels in *ttg2* mutant seed mirrored the flavonol pattern in being reduced to almost undetectable levels in 5DAP, but, in this case, this was true for both Col-0 and L*er* (Figure 5.6). Taken together this data suggests that *TTG2* acts as a major regulator of transcriptional networks in the FBP, similar to the roles played by TT2, TT8 and TTG1 (Johnson, 2002; Debeaujon *et al.*, 2003; Lepiniec *et al.*, 2006).

Interestingly, the impact on flavonoid levels of some mutants was not always consistent with their roles described in the literature. For example, soluble procyanidin levels in *ban* were higher than control levels. Since the Col-0 *ban* line used in the present work allele was a knockdown mutant (see Chapter3: section 3.2.4), the expectation was for a reduced PA level rather than complete PA elimination as reported for the mature seed of a confirmed *ban* null mutant in the Ws-2 ecotype (Routaboul *et al.*, 2006). However, the observed increase in PA levels in the Col-0 *ban* line was unexpected; one possible explanation is that production of low levels of PAs from the known central FBP pathway triggers activation of another as yet uncharacterised PA synthesis pathway that results in over-production of PAs. The same analysis could be made about flavonol levels in *fls*. However, this statement cannot be made unless true knockout mutants are generated for *BAN, FLS*. Regarding *anl1*, which had similar A1 levels to WT control, the results seemed to contradict with our understanding of the FBP, and with the RT-PCR results that showed that anl1 was a true knockout. So, a possible explanation for not having significantly reduced levels of A1 –as predicted by the FBP- would be that A1 could be synthesised through another pathway, independent of ANL1. However, this statement needs further investigation.

In fact, and in relation to *fls*, the flavonols quercetin and kaempferol (Wisman *et al.*, 1998;

Routaboul *et al.*, 2006) and PAs (Routaboul *et al.*, 2006) were reported to have lower values in *fls-1* than in Col-0 controls. This indicates that FLS is not the only enzyme involved in the production of flavonols and PAs. Collectively, this data, along with the fact that *fls* mutant used in this present work was a knockdown, could explain the similar levels of flavonols between wild-type and *fls*.

5.4 Conclusions

This chapter aimed to investigate the link between products of the FBP and the interploidy cross effect by directly measuring flavonoids in seeds at a time-point previously established as pivotal for successful seed development. Three classes of flavonoids – flavonols, anthocyanins (A1) and PAs – and the auxin IAA, were analysed by LC-ESI-QQQ-MS/MS in 5DAP (heart stage) seeds of wild-type Col-0 and L*er* and various FBP mutants. Some general conclusions can be drawn:

1) 5DAP seed of the L*er* ecotype contain much higher levels of flavonols and PAs than the Col-0 ecotype, but both ecotypes have roughly similar levels of anthocyanin A1. Since a L*er* seed parent improves seed survival in 2xXCol4x crosses (see Chapter 3: sections 3.2.2.1 and 3.2.2.2) this is consistent with high flavonol/PA levels mitigating the Col-0 killer effect.

2) The null *tt4* mutant in L*er* completely abolishes all three classes of flavonoids. This is also the case for PAs and A1 in Col-0 *tt4*, but while flavonol accumulation is dramatically reduced to 17% of wild-type, it is not eliminated suggesting an alternative pathway exists for their synthesis. More significantly, since *tt4* seed parents in both Col-0 and L*er* dramatically improve seed survival in 2xXCol4x crosses (see Chapter 3: sections 3.2.2.1, 3.2.2.2, 3.2.6.1 and 3.2.6.2) no or low flavonoid levels are positively correlated with mitigating the Col-0 killer effect. This contracts conclusion 1 above.

3) *ttg2* had different impacts on flavonol levels in the two ecotypes; these were at almost wild-type levels in Col-0, whilst being dramatically reduced in L*er*. Interestingly, PAs and A1 were abolished in both ecotypes suggesting that *TTG2* acts as a major regulator of transcriptional networks in the FBP, similar to the roles played by *TT2*, *TT8* and *TTG1*.

4) Seeds of the Col-0 ecotype had much higher levels of IAA at 5DAP compared to L*er*. Thus a low auxin level was positively correlated with improved seed survival in

2xXCol4x crosses. However, the data presented here found no convincing connection between IAA levels and the FBP pathway.

5) The presence of largely wild-type levels all three measured classes of flavonoids in the Col-0 *fls,* and *ban* alleles suggest that these are knockdown rather than knockout mutants, or an alternative biosynthesis pathway in case of *anl1*.

5.5 Limitations and future work

Having a range of T-DNA insertion FBP mutants (full knockouts) in just the Col-0 ecotype and not the L*er* ecotype was a limitation of the study, thus to solve inconsistency on selection of mutants it would have been useful to have groups of FBP mutants in both the Col-0 and L*er* ecotypes. Moreover, and due to the stage of the seeds (5 DAP), establishing a full profile of anthocyanins and PAs was not possible; therefore better to cover different stages of development. Developing protocols that would cover a wide range –if not all - the subsets of anthocyanins and PAs are therefore highly desirable.

Chapter 6: The effect of FBP mutations on auxin distribution in diploid and tetraploid crosses

6.1 Introduction

Plant hormones or growth regulators play an important part in all stages of plant development starting with embryogenesis right up to senescence. These are small molecules that are produced from a range of metabolic pathways (Cheng *et al.*, 2013; Miransari and Smith, 2014). At the beginning of the last century, plant hormone studies led to the detection of auxin (IAA), abscisic acid (ABA), brassinosteroids (BRs), cytokinins (CK), ethylene (ET), gibberellins (GA), jasmonic acid (JA), salicylic acid (SA), and the latest discovery, strigolactones (SLs) (Kim *et al.*, 2005; Santner *et al.*, 2009; Cheng *et al.*, 2013). Usually, the effects of plant hormones are found on or near to the location of biosynthesis, or they can be mobile between various tissues. There are many different kinds of hormone crosstalk mechanism. It is known that it is at the level of gene expression that hormone signalling pathways communicate. It has been suggested that hormones might regulate sensitivity (hormone response), synthesis (hormone levels), and the transport (hormone distributions) of other hormones (Kim *et al.*, 2005; Santner *et al.*, 2009; Cheng *et al.*, 2013; Bellini *et al.*, 2014).

This chapter describes the use of the auxin reporter DR5::GFP to better understand the part played by flavonoids in auxin transport, how flavonoids are transported into the vacuole and to identify regulatory factors that control flavonoid gene expression. The experimental approach involved assaying GFP in selected FBP mutants carrying the reporter.

6.1.1 The biosynthesis of IAA

Indole Acetic Acid (IAA) was the first natural auxin discovered. IAA generates from the amino acid tryptophan (Trp) (Normanly *et al.*, 1995; Tivendale *et al.*, 2014). Tivendale *et al.* (2014) suggested five IAA biosynthetic pathways: a Trp-independent IAA biosynthetic pathway, and four interconnected Trp-dependent pathways. The indolic 3-pyruvic acid (IPA) pathway includes the most related enzymes in the biosynthesis of auxin, which are YUC (flavin-monooxygenases) and TAA/TAR (tryptophan amino-transferases) (Mashiguchi *et al.*, 2011; Stepanova *et al.*, 2011; Won *et al.*, 2011). Cheng

et al. (2006) suggested that the rate-limiting stage of the IPA pathway was catalysed by YUCCA (YUC) proteins. The *A. thaliana* genome contains 11 YUC genes, 4 of which (YUC1, YUC4, YUC10, and YUC11) were expressed in the embryo in an over- lapping way (Cheng *et al.*, 2007).

6.1.2 Auxin role in the cellularisation of endosperm

Auxins, including indole-3-acetic acid (IAA), which is the most prevalent natural auxin, convey molecular signals for plant growth and development, for example, bud outgrowth. Auxins are essential for cell division and cell expansion (Perrot-Rechenmann, 2010; Cheng *et al.*, 2013), patterning during embryogenesis, organogenesis, and the formation of vascular tissue, the growth direction, and long-distance signalling (Leyser, 2011; Petrasek and Friml, 2009). Along with unregulated bulk movement in the phloem, auxin has a unique mode of transport (basipetally), with cell-to-cell transfer from source tissues such as the shoot apex, young leaves and flowers toward the root apex, which is mediated by auxin carriers, like AUX/LAX, PIN and P-Gluco Protiens (PGP) (Petrasek and Friml, 2009). Eight PIN proteins have been isolated so far in *A. thaliana*, with specific sub-cellular localisations. PIN5, PIN6 and PIN8 are believed to regulate auxin exchange between the endoplasmic reticulum and the cytosol, while PIN1, PIN2, PIN3, PIN4 and PIN 7 are plasma membrane auxin efflux carriers (Petrasek and Friml, 2009).

More importantly for the present work, flavonoids are known to affect the distribution of auxin (and thus have been utilised as the natural auxin transport inhibitors), although the mechanism modulating auxin transport is still unclear (Peer and Murphy, 2007). The flavonoids accumulate in the endothelium layer (Devic *et al.*, 1999; Abrahams *et al.*, 2002; Abrahams *et al.*, 2003; Debeaujon *et al.*, 2003; Kleindt *et al.*, 2010). However, flavonoids possibly interact directly with auxin transporters, for instance, P-glycoprotein1 (PGP1), PGP4 and PGP19 (Peer and Murphy, 2007; Santelia *et al.*, 2008). Furthermore, it seems that through the modulation of PIN regulation, flavonoids can have indirect effects on auxin transport (Peer *et al.*, 2011). The inhibiting of auxin transporters activity is via inhibition of ATPase, phosphatase or kinase activity (Peer *et al.*, 2011; Peer and Murphy, 2007). Some flavonols such as quercetin and kaempferol have been shown to inhibit polar auxin transport (Brown *et al.*, 2001; Peer and Murphy, 2007; Santelia *et al.*, 2008).

6.1.3 The auxin reporter construct

The movement of auxin in a tissue cannot be followed directly; therefore the investigators of auxin movement have examined auxin by making use of GFP fusions (Huang *et al.*, 2010) or through synthetic promoters based on auxin-inducible genes (Petrasek and Friml, 2009), or through the use of antibodies to label the PIN auxin efflux carriers (Benkova *et al.*, 2003). The synthetic promoter DR5 was made through creating site-directed mutations on the 5' end of the 11bp auxin response element (AuxRE) D1-4 (5'-CCTCGTGTCTC-3') in the *Gretchen Hagen* GH3 promoter, which is auxin -induced (Ulmasov *et al.*, 1997).

Ulmasov *et al.* (1997) showed that DR5 consisted of tandem direct repeats of the AuxRE D1-4 and demonstrated greater auxin responsiveness compared to the natural composite AuxRE. These promoter elements were shown to be the target sequence for auxin response transcription factor 1 (ARF1) *in vitro* (Ulmasov *et al.*, 1997; Tiwari *et al.*, 2003). Moreover, an interaction between ARF1 and Aux/IAA proteins was identified in a yeast two-hybrid system. These Aux/IAA proteins repress auxin-inducible genes when dimerized with ARF1 (Ulmasov *et al.*, 1997; Tiwari *et al.*, 2001). Upon increase in auxin levels, auxin binds to the AUX/IAA repressor, thus destabilising it and allowing TRANSPORT INHIBITOR RESPONSE 1 (TIR1), which is an auxin receptor on the SCF^{TIR1} complex, to bind it –along with the AUX/IAA- and to degrade it –AUX/IAA- through ubiquitination mediated by the F-box protein subunit of the SCF^{TIR1} ubiquitin-ligase complex (Dharmasiri *et al.*, 2005; Kepinski and Leyser, 2005; Woodward and Bartel, 2005a, 2005b; Guilfoyle, 2007). Guilfoyle (2007) point out that the ARF1, which is bound to the DR5AuxRE, stays bound to transcriptional repressors Aux/IAA in low concentrations of auxin. However, high auxin concentration triggers the SCF^{TIR1} complex, which targets Aux/IAA repressors for degradation and consequently releases the transcriptional repression of DR5AuxRE furthermore allowing expression of the conjugated auxin-inducible gene (Figure 6.1).

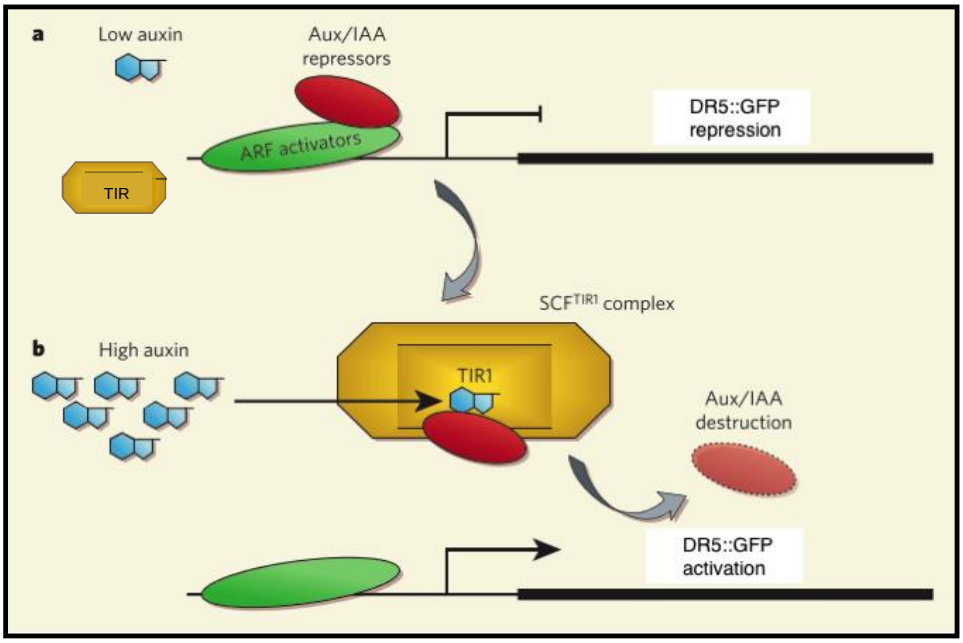


Figure 6.1: A model of DR5::GFP's action illustrating auxin signal. a) At low concentrations of auxin, Aux/IAA repressors are bound to ARF, which itself is bound to DR5 AuxRE. **b**) At high concentrations of auxin, the TIR1 subunit of the SCF^{TIR1} complex binds to auxin. The SCF^{TIR1} complex ubiquitinates the Aux/IAA repressors to allow degradation by the proteasome; and this releases the transcriptional repression of the DR5 AuxRE (adapted from Guilfoyle, 2007).

6.1.4 Hypothesis and aims

In our hypothesis, in seed generated by a balanced genome cross (2xX2x, 4xX4x) the onset of endosperm cellularisation at the appropriate developmental time point, i.e., heart stage/5DAP maternal is regulated by a signal originating in the maternal integument layers and transported into the peripheral endosperm through the endothelium layer. In Col2x XCol4x crosses endosperm cellularisation is late or absent resulting in seed death (the Col-0 killer effect). This effect can be counteracted by substituting the 2x seed parent with a 'rescuing' ecotype, such as wild-type L*er*, or a Col-0 or L*er* seed parent carrying one of a number of FBP mutations, such as *tt4* (see Chapter 3).

The current working hypothesis is that auxin is the maternal cellularisation signal based on 1) the known role of auxin in regulating cell division and 2) since flavonols can inhibit polar auxin transport, flavonols synthesised in the endothelium by the FBP could modulate polar auxin transport across the integuments to influence the timing and amplitude of the maternal cellularisation signal. This hypothesis would explain the data

described in Chapters 3 and 5 showing that FBP mutants, such as *tt4,* that block flavonoid biosynthesis, initiate cellularisation around the critical 5DAP window resulting in seed rescue in 2xX4x Col-0 crosses. The absence of inhibitory flavonols would increase and/or provide earlier auxin transport from the maternal integuments into the peripheral endosperm to initiate cellularisation.

The experimental approach was to produce *A. thaliana* lines combining the DR5::GFP reporter gene and various *tt* mutants to investigate the effect of blocking the FBP on the accumulation of auxin in the seed coat.

Therefore, there are two major aims of this chapter:

1. To investigate auxin response localisation and intensity in the wild type seed generated by balanced and interploidy crosses.
2. To examine how FBP mutations affect the accumulation of auxin response in the seed balanced and interploidy crosses. The FBP mutants were: *tt4*, *ttg2*, *fls* and *ban.* Both *tt4* and *ttg2* rescue seed lethality in 2xXCol4x crosses as seed parents, while *fls* and *ban* seed parents do not rescue.

6.2 Results

6.2.1 IAA levels in developing seeds of wild-type and FBP mutants

Given the established ability of flavonoids to interfere with the polar transport of auxin and the possibility that this might be how the FBP regulates endosperm development, LC-ESI-QQQ-MS/MS was used to measure IAA levels in wild-type and FBP mutant seed. The FBP mutants, *tt4*, *anl1*, and *ban*, had significantly lower "relative" IAA levels compared to the Col-0 controls. In contrast, IAA levels in *fls* and *ttg2* in Col-0 were similar to wild-type. The picture was different for the L*er* ecotype where *ttg2* had significantly higher "relative" IAA levels, while *tt4* was unchanged compared to wild-type (Figure 6.2).

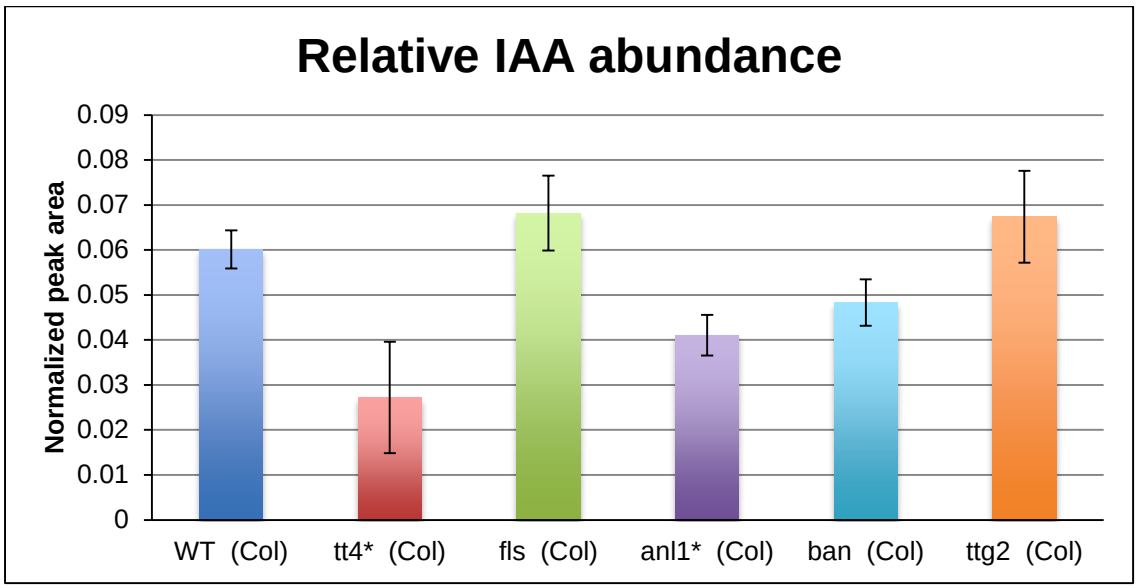


Figure 6.2: Relative IAA abundance in a range of FBP mutants and their control ecotypes in *A. thaliana* seed at 5DAP. Mean values ± standard error of three independent measurements except *fls* have one sample. Bars labelled * were *tt* mutants that displayed statistically significantly different from the control data ($P \leq 0.05$).

6.2.2 The signals of auxin in the integuments varied according to *tt* homozygous mutant and paternal genome size in Col-0 ecotype as well as development stage

To understand how auxin might force endosperm cellularisation, a comparison of seeds was made at four critical developmental stages through: a) seed resulting of DR5::GFP/FBP mutant (2X) - maternal parent- (Unfertilised). b) seed resulting of crosses between DR5::GFP/FBP mutant (2X) and Col2x pollen parents. c) seed resulting of crosses between DR5::GFP/FBP mutant (2X) and Col4x pollen parents.

The developmental stages were at 3DAP (globular embryo), 5DAP (heart embryo, endosperm cellularisation), 7DAP (torpedo embryo) and 9DAP (late torpedo stage). IMARIS software was used to collect GFP fluorescence quantitative data of the Z-stacks of the seed coat and endosperm images manually without the funicular and micropylar regions. Due to very high levels of GFP signal, these two regions were excluded from the manual extrusions as this may have skewed the data.

The 9DAP sample sizes were generally a little lower because when attempting to load and analyse Z-stacks with sliced 40-50 (> 150mb), the IMARIS software frequently crashed with larger seeds.

6.2.2.1 A qualitative analysis of the differences in auxin accumulation in the integuments of diploid and tetraploid crosses at various developmental stages

At different seed development stages (3, 5, 7 and 9 DAP), auxin accumulation varied enormously in the seed coat tissues of different mutants in diploid (DR5::GFP/FPB mutants (2x)XCol2x) and interploidy (DR5::GFP/FBP mutants (2x)XCol4x)crosses.

At 0DAP (unfertilised seed), there was no auxin signal in the seed coat, but there was some GFP signal in the funiculus, except in DR5::GFP/*ttg2* seeds; moreover, DR5::GFP/*tt4* seeds showed higher levels of GFP (Figure 6.3).

At 3DAP, there was strong GFP expression DR5::GFP/Col2xXCol2x wild-type seed and –at a lower level- in DR5::GFP/*ban*XCol2x crosses (Figure 6.4.A and D) while in DR5:: FP/*ban*XCol4x GFP expression was stronger than DR5::GFP/Col2xXCol4x (Figure 6.5.D).

Interestingly, at later developmental stages (5, 7 and 9 DAP), the accumulation of auxin in seed coat was regularly observed to increase in DR5::GFP/Col2xXCol2x wild type, DR5::GFP/*tt4*XCol2x, and DR5::GFP/*ban*XCol2x seeds. While in DR5::GFP/Col2xXCol4x, DR5::GFP/*tt4*XCol4x, and DR5::GFP/*ban*XCol4x seeds, the accumulations of auxin in seed coat were regularly increased at 5 and 7 DAP. But at 9 DAP GFP expression was very strong in DR5::GFP/Col2xXCol4x, and DR5::GFP/*ban*XCol4x seeds coat.

At 5DAP, *ban* mutant crosses (DR5::GFP/*ban*XCol2x) had a high auxin-reporting GFP signal in the seeds coat (Figure 6.4.I), however WT (DR5::GFP/Col2xXCol2x), presented a lower auxin levels than DR5::GFP/*ban)*XCol2x seeds in the seed coat (Figures 6.4.F and I).

Moreover, DR5::GFP/*ban*XCol4x and DR5::GFP/Col2xXCol4x crosses exhibited a strong GFP fluorescence within clearly delineated cell borders (Figure 6.5.F and I) whereas DR5::GFP/*tt4*XCol4x had a diffuse GFP signal (Figure 6.5.G).

Clearly, DR5::GFP/*ttg2*XCol2x, DR5::GFP/*fls*XCol2x, DR5::GFP/*ttg2*XCol4x and DR5::GFP/*fls*XCol4x crosses showed no auxin expression in the seed coat as judged by the absence of GFP signal.

At 7DAP, DR5::GFP/Col2xXCol2x, DR5::GFP/*ban*XCol2x, DR5::GFP/*tt4*XCol2x, DR5::GFP/Col2xXCol4x, DR5::GFP/*ban*XCol4x and DR5::GFP/*tt4*XCol4x crosses had an established pattern of auxin level in the seed coat that continued to intensify (Figures 6.4 K, N, L and 6.5 K, N, L, respectively).

Auxin signal patterns in seed coat of DR5::GFP/*fls*XCol2x, DR5::GFP/*ttg2*XCol2x, DR5::GFP/*fls*XCol4x and DR5::GFP/*ttg2*XCol4x mutants crosses were consistently little to none.

In DR5::GFP/*ban*XCol2x, DR5::GFP/Col2xXCol2x, DR5::GFP/*ban*XCol4x and DR5::GFP/Col2xXCol4x crosses the overall pattern of auxin accumulation continued and intensified at 9DAP (Figures 6.4.S, P and 6.5.S, P). In DR5::GFP/*tt4*XCol2x cross, auxin accumulation continued and increased at 9DAP (Figure 6.4 Q) while in DR5::GFP/*tt4*XCol4x cross auxin level continued and increased at 7DAP(Figure 6.5.L) then decreased at 9DAP (Figure 6.5.Q).

On the other hand, DR5::GFP/*fls*XCol2x, DR5::GFP/*ttg2*XCol2x, DR5::GFP/*fls*XCol4x and DR5::GFP/*ttg2*XCol4x crosses had a consistently low auxin concentration that only appeared in the funicular tissues.

Furthermore, the pattern of auxin GFP signal in DR5::GFP/*ban*XCol2x, DR5::GFP/Col2xXCol2x and DR5::GFP/*tt4*XCol2x crosses and the absence of auxin GFP signal in DR5::GFP/*fls*XCol2xand DR5::GFP/*ttg2*XCol2x was generally constant (Figures 6.4) and thus replicated what was observed in the in DR5::GFP/*ban*XCol4x, DR5::GFP/Col2xXCol4x, DR5::GFP/*tt4*XCol4x, DR5::GFP/*fls*XCol4x and DR5::GFP/*ttg2*XCol4x crosses (Figure 6.5).

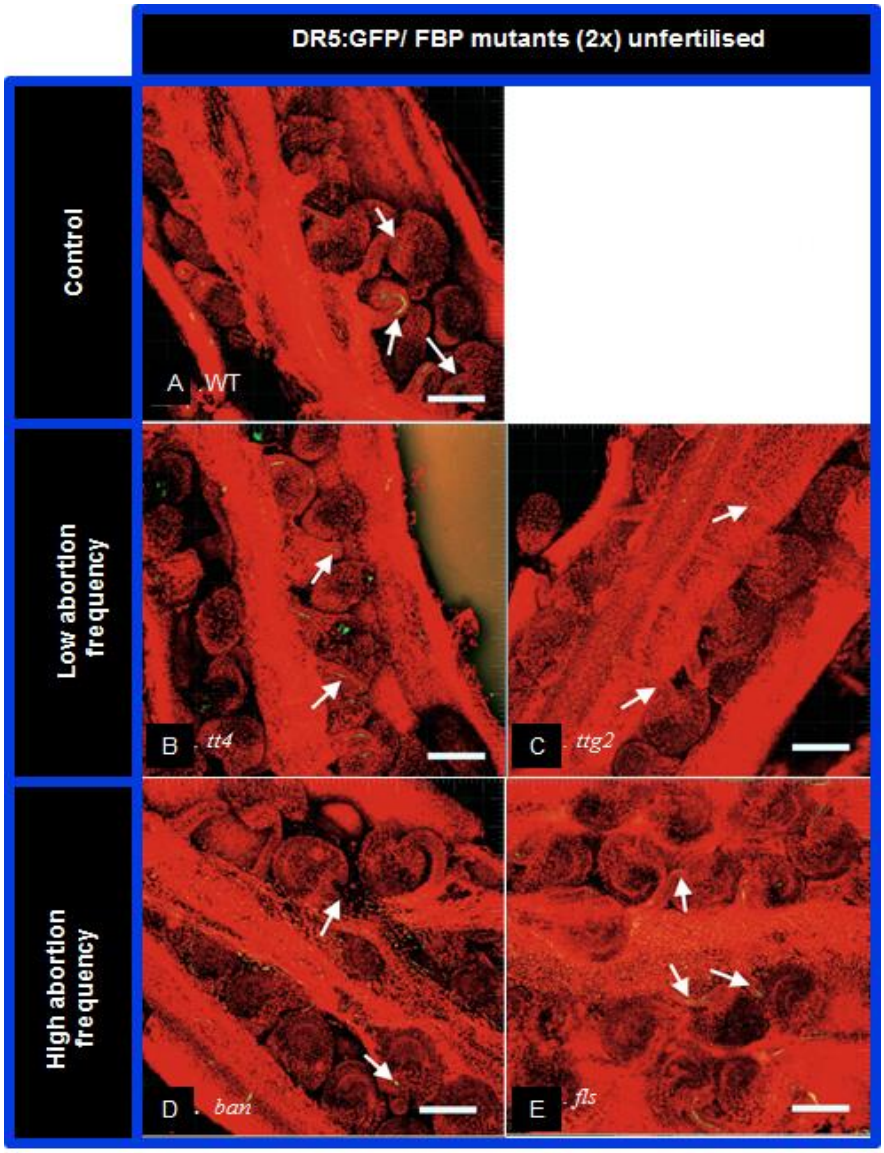


Figure 6.3: Confocal laser-scanning photomicrographs of unfertilised seed, diploid, *A. thaliana* Col-0 WT and mutant seeds, containing DR5::GFP auxin response reporter. White arrows illustrate funicular tissues. Scale bar = 100µm. Please note that mutant names in the subfigures correspond to DR5::GFP/FBP mutants; e.g. *tt4* is DR5::GFP/*tt4*.

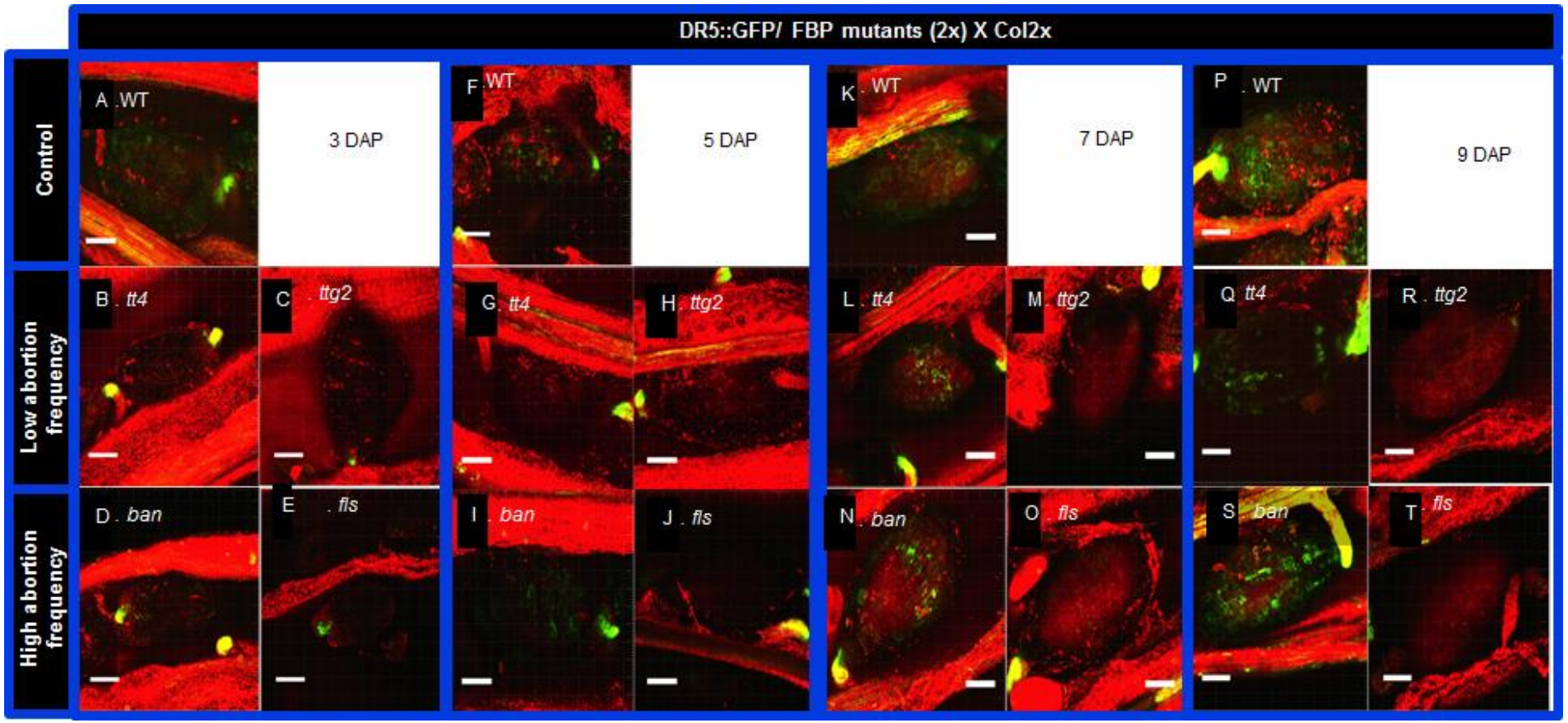


Figure 6.4: Confocal laser-scanning photomicrographs of seeds resulting of maternal DR5::GFP/Col2x (WT) and paternal Col2x, as well as maternal DR5::GFP/FBP mutants and paternal Col2x (diploid crosses), at 3, 5, 7 and 9 DAP. A, B, C, D and E at 3DAP; F, G, H, I and J at 5DAP; K, L, M, N and O at 7 DAP; P, Q, R, S and T at 9 DAP. Scale bar = 100µm. Please note that mutant names in the subfigures correspond to DR5::GFP/FBP mutants; e.g. *tt4* is DR5::GFP/*tt4*.

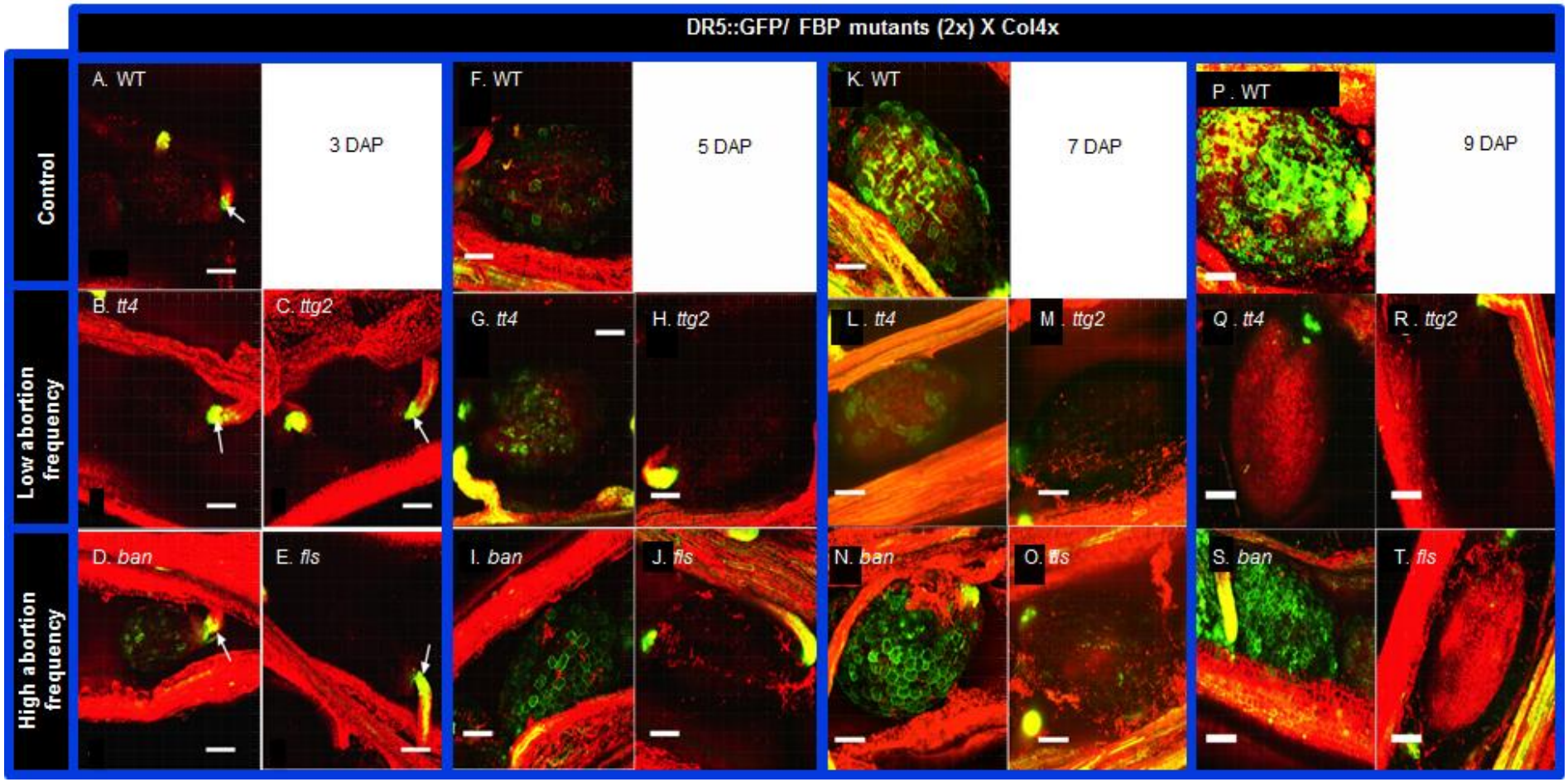


Figure 6.5: Confocal laser-scanning photomicrographs of seeds resulting of maternal DR5::GFP/Col2x (WT) and paternal Col4x, as well as maternal DR5::GFP/FBP mutants and paternal Col4x (interploidy crosses), at 3, 5, 7 and 9 DAP. A, B, C, D and E at 3DAP; F, G, H, I and J at 5DAP; K, L, M, N and O at 7 DAP; P, Q, R, S and T at 9 DAP. White arrows illustrate GFP signal in funicular tissues. Scale bar = 100µm. Please note that mutant names in the subfigures correspond to DR5::GFP/FBP mutants; e.g. *tt4* is DR5::GFP/*tt4*.

6.2.2.1.1 Is auxin GFP fluorescence present in the inner or outer integuments?

In this section, two mutants were chosen to study the localisation of the DR5::GFP signal in the 5DAP seeds: a) *ban*, as a representative for lethal phenotype, and b) *tt4* as an example of rescued seeds (at least in L*er* ecotype). Results showed that the distribution of auxin between tissues is different between the control (DR5::GFP/Col2xXCol4x) and mutant seeds (DR5::GFP/*ban*XCol4x and DR5::GFP/*tt4*XCol4x); however, results were not totally clear: as the laser penetrates the deeper tissues, the emission of light diminishes as it passes through the cell layers. For this reason, it is difficult to separate the layers of the integuments unambiguously. However, Figure 6.6 shows some representative Z-stack slices where auxin accumulation in the integument layers can be discerned.

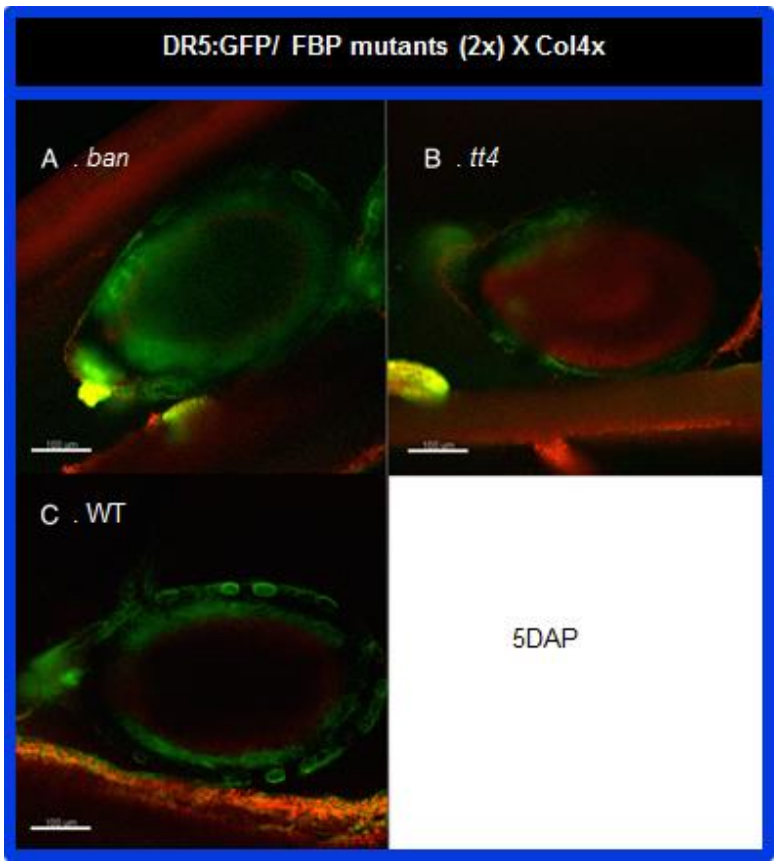


Figure 6.6: Confocal micrographs of *A. thaliana* 5DAP seeds containing DR5::GFP construct, showing spatial auxin response expression. Seeds resulted from an interploidy cross between maternal DR5::GFP/Col2x(WT) and Col4x pollen, DR5::GFP/FBP mutants maternal plants and Col4x pollen. A. DR5::GFP/*ban*XCol4x, **B.** DR5::GFP/*tt4*XCol4x, **C.** DR5::GFP/Col2xXCol4x. Please note that there was a slight variation in the accumulation of auxin in the individual seed coat layers. The distribution of auxin GFP fluorescence in WT crosses (DR5::GFP/Col2xXCol4x) was more similar to DR5::GFP/*ban* mutant crosses in the seed coat. The distribution of GFP fluorescence appeared at the outer integument layers and also near or at the endothelial layer permitting discernment of individual cells. However, in seeds resulting from crosses containing maternal DR5::GFP/*tt4*, GFP fluorescence was observed near or in the inner integument layers. Scale bar = 100µm. Please note that mutant names in the subfigures correspond to DR5::GFP/FBP mutants; e.g. *tt4* is DR5::GFP/*tt4*.

6.2.2.2 A quantitative analysis of the different auxin accumulations in the integuments of seeds resulting from crosses between the diploid and tetraploid paternal pollen with the maternal WT and FBP mutants

Fluorescence intensity (µm^{-3}) of the GFP proxy was calculated in order to quantify the level of auxin in the integument layers. IMARIS software was used to measure the values of the sum of the whole green channel fluorescence intensity (µm^{-3}) and the Z-stacks were used to obtain the corresponding volume of the endosperm and the whole seed. Calculations of the strength of fluorescence (µm^{-3}) in integument and endosperm were made using the same method. The integument layer intensity was calculated according to the following equation:

$$\text{Intensity } (\mu m^{-3}) = \frac{\text{whole seed intensity sum - endosperm intensity sum}}{\text{whole seed volume - endosperm volume}}$$

Log_{10} transformation was used to normalise the data, as there was very sharp magnitudinal difference of intensity of GFP fluorescence between mutant diploid and interploidy crosses (DR5::GFP/*ban*XCol2x, DR5::GFP/*ttg2*XCol2x, DR5::GFP/*ban*XCol4x, DR5::GFP/*ttg2*XCol4x for example). The retrieved data was summarised in clustered bar charts (Figure 6.7.A and B), which mirrored the above observations.

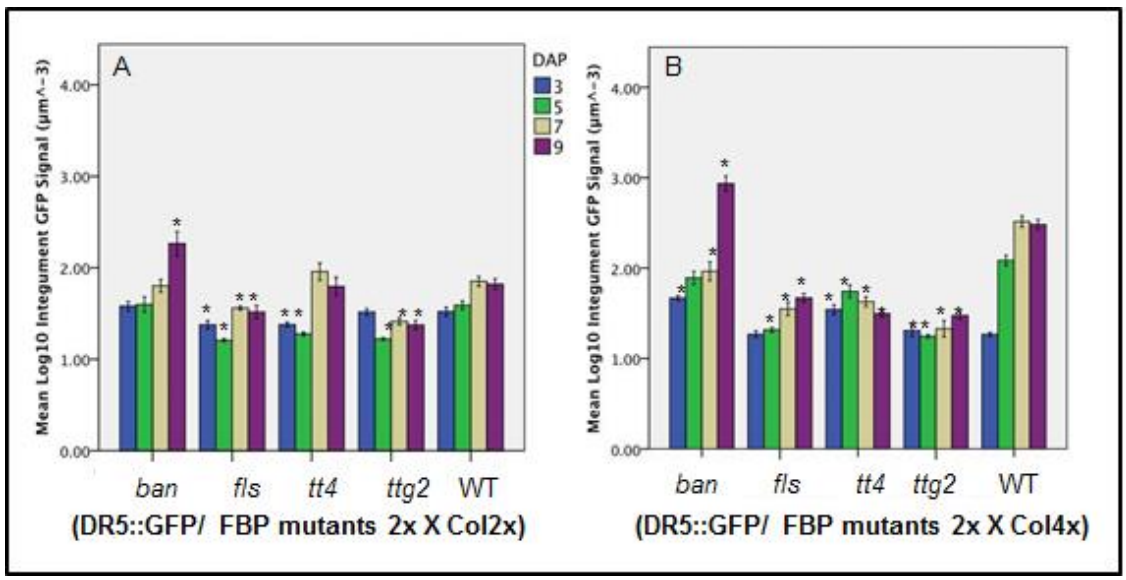


Figure 6.7: Graphical representation of the Log_{10} [GFP intensity] in seeds integuments at different developmental stages, resulting from diploid and interploidy crosses. GFP signal was generated by the auxin reporter construct DR5::GFP. A. Seed resulting from diploid crosses (DR5::GFP/FBP mutants (2x) X Col2x). **B.** Seeds resulting from interploidy crosses (DR5::GFP/FBP mutants (2x) X Col4x). A strong auxin signal was seen in the integuments layers in tetraploid crosses. **N**= 10 seeds. Bars labelled * were shown *tt* mutants that were statistically significantly different from the control data ($P \leq 0.05$). Please note that mutant names in the subfigures correspond to DR5::GFP/FBP mutants; e.g. *tt4* is DR5::GFP/*tt4*.

As development progressed, the level of GFP fluorescence (auxin response accumulation) in seeds resulting from DR5::GFP/*ban*XCol2x, DR5::GFP/*tt4*XCol2x and DR5::GFP/Col2xXCol2x crosses increased, while other mutant crosses (DR5::GFP/*ttg2*XCol2x and DR5::GFP/*fls*XCol2x) failed to demonstrate any clear increase in auxin accumulation. At all developmental stages (3, 5, 7 and 9 DAP) the quantitative data shows auxin GFP fluorescence in tetraploid crosses was greater than in diploid crosses. Furthermore, the level of auxin expression integuments in

DR5::GFP/*ban*XCol2x and DR5::GFP/*ban*XCol4x mutant crosses were significantly higher than (WT) DR5::GFP/Col2xXCol2x and DR5::GFP/Col2xXCol4x crosses at all stages of development.

6.2.2.2.1 No auxin signal observed in the endosperm area

Results showed that seeds had a very low level of GFP fluorescence in their endosperm, in contrast to the high levels in the integument layers (Figure 6.8).

It is hard to determine from the images, but the patterns in increasing GFP fluorescence in the endosperm identified by the quantitative data (Figure 6.9.A and B) are generally the same as that in the integuments (Figure 6.7.A and B), albeit at an intensity that is significantly lower (N.B. this observation is exaggerated by the Log_{10} data).

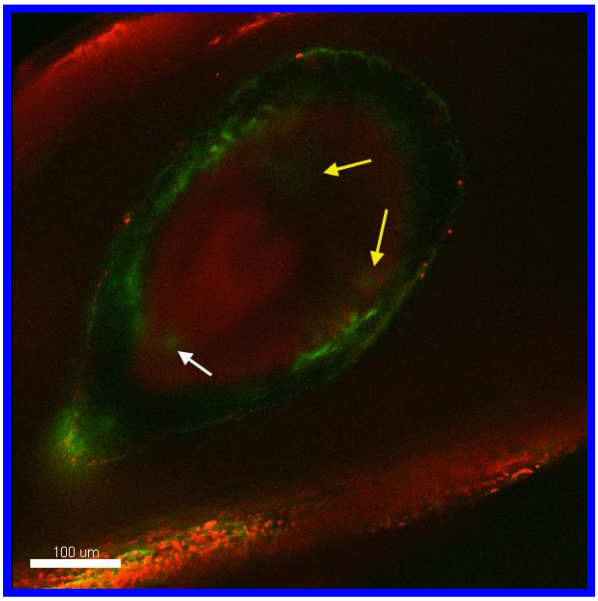


Figure 6.8: A single Z-stack slice of the DR5::GFP/Col2xXCol2x showing the very low levels of GFP fluorescence in the endosperm of seeds at 7DAP (post cellularisation). N.B. The intensity of the green channel signal (Gain) was amplified to highlight the GFP fluorescence difference between the integument layers and endosperm. Yellow arrows highlight the supposed area of interference caused by out-of-focus light from another focal plane. The embryo is noticeable at late heart/early torpedo stage. GFP fluorescence in the suspensor is highlighted by a white arrow, and is consistent with Petrásek and Friml's (2009) work, which shows the signal and accumulation of auxin within the suspensor and embryo. Scale bar = 100 μm.

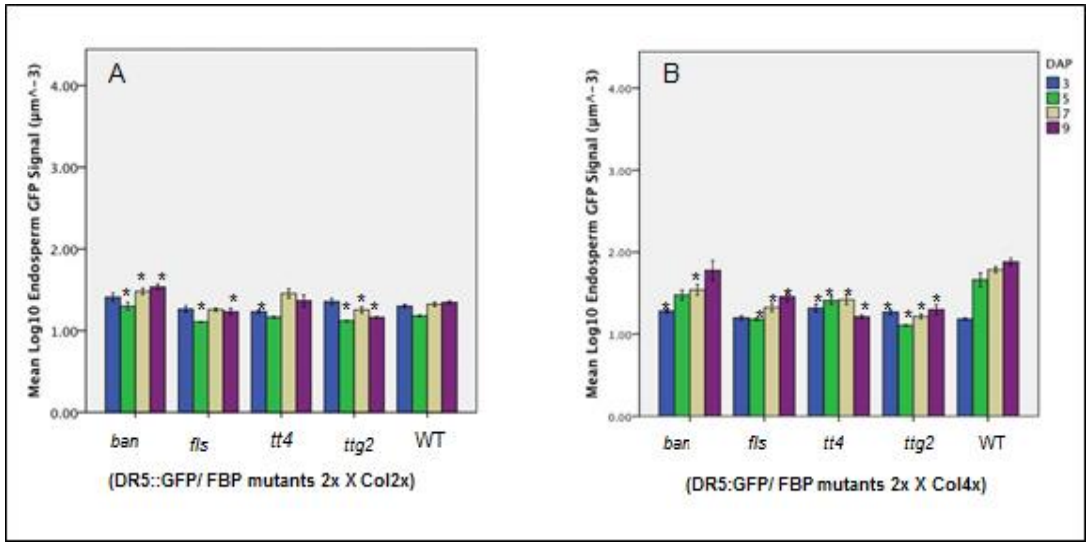


Figure 6.9: Graphical representation of the Log_{10} [GFP intensity] in seeds endosperm at different developmental stages, resulting from diploid and interploidy crosses. GFP signal was generated by the auxin reporter construct DR5::GFP. A. Seed resulting from diploid crosses (DR5::GFP/FBP mutants (2x) X Col2x). **B.** Seeds resulting from interploidy crosses (DR5::GFP/FBP mutants (2x) X Col4x). **N**= 10 seeds. Bars labelled * were shown *tt* mutants that were statistically significantly different from the control data ($P \leq 0.05$). Please note that mutant names in the subfigures correspond to DR5::GFP/FBP mutants; e.g. *tt4* is DR5::GFP/*tt4*.

6.2.2.3 Parental origin and allele number of DR5::GFP in the genome does not affect the auxin response levels in WT plants involved in diploid and interploidy crosses

An initial experiment (using a small sample size) was conducted to determine whether the level of GFP varied between self-pollinated DR5::GFP/Col2x (the offspring genome inherited two copies of DR5::GFP) and the controlled-pollinated DR5::GFP/Col2xXCol2x (one copy of DR5::GFP). No significant difference in the GFP fluorescence in the maternally derived seed coat tissues was revealed when a Welch Two Sample t-test was conducted (p=0.44, 95% confidence interval). The difference in the level of GFP fluorescence was negligible and not significant between Col2xXDR5::GFP 2x and Col2xXDR5::GFP 4x.

6.2.2.4 The changes in vacuole structure in the endothelium layer of some tt mutants in the Col-0 ecotype

To further investigate the variation of auxin localisation and accumulation in the seed coat, especially in endothelium layer; the immature seeds were stained with toluidine blue (TB) after fixing and cut into 5 µm thick slices. The structure of the endothelial vacuolar

cell was clearly observed in each mutant. In DR5::GFP/*tt4*XCol4x, TB signals were observed outside small vacuoles. In contrast, a large central vacuole was detected in immature seeds of DR5::GFP/Col2xXCol4x, DR5::GFP/*ban*XCol4x, DR5::GFP/*fls*XCol4x and DR5::GFP/*ttg2*XCol4x (Figure 6.10).

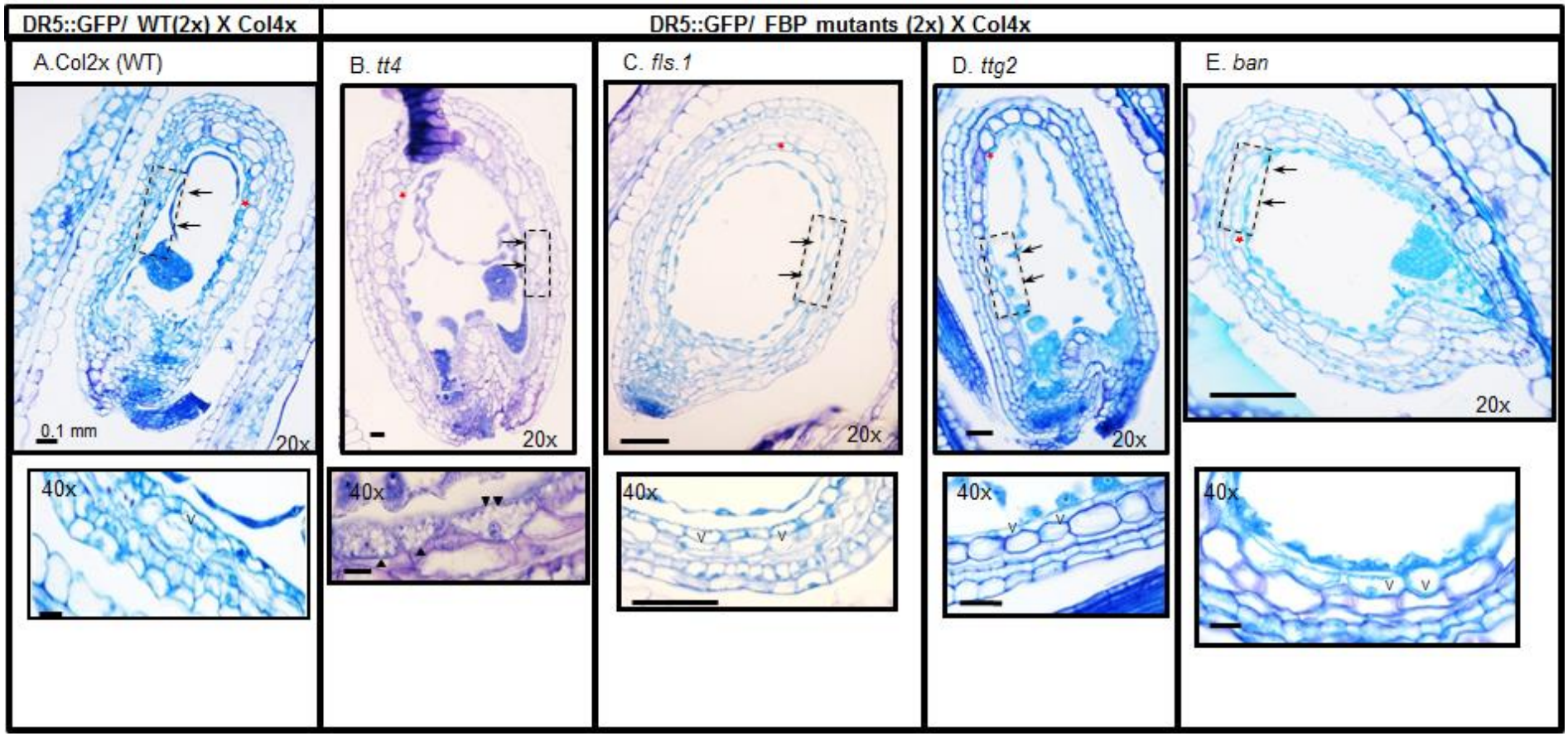


Figure 6.10: Toluidine blue staining of a 5 µm thick section of immature seeds (5 DAP) in interploidy crosses. Seeds resulting from interploidy crosses (DR5::GFP/FBP mutants (2x) X Col4x). Endothelium layers are arrowed in both panels at 20x magnification. Enlarged images of boxed regions were taken at 40x magnification, including single cells. A larger vacuole is indicated (**v**) and some small vacuolar-like structures (arrowheads) were found in DR5::GFP/*tt4*XCol4x. Scale bar: 0.1mm. Please note that mutant names in the subfigures correspond to DR5::GFP/FBP mutants; e.g. *tt4* is DR5::GFP/*tt4*.

6.3 Discussion

The purpose of these studies was to analyse IAA level in developing seeds at 5DAP; and to investigate the localisation of auxin using the DR5::GFP (Ulmasov *et al.*, 1997) auxin-response reporter as development progressed.

6.3.1 Analysing auxin levels in developing seeds of wild-type and FBP mutants at 5 DAP

As previously discussed flavonols are known to interfere with aspects of auxin activity, most notably polar auxin transport (Peer *et al.*, 2004; Lazar and Goodman, 2006). Interestingly, the present work found a strong negative correlation between flavonol and IAA levels in 5DAP seed of Col ecotype (see Chapter5: Figure 5.4; Figure 6.2).

There are a number of possible explanations. 1) Reduced polar auxin transport away from the source toward its sink by the relatively high levels of flavonols (kaempferol and flavonol glycosides) in L*er* seeds (see Chapter5: Figure 5.2) could negatively regulate auxin synthesis via a feedback loop. 2) Flavonols could directly supress auxin synthesis at source. The results from the *tt4* mutants may help decide between these two possibilities. The Col-0 *tt4* allele results in the almost complete elimination of flavonols (see Chapter5: Figure 5.4); auxin measurements in Col-0 *tt4* showed low levels (Figure 6.2). Thus, elimination of flavonols via *tt4* resulted in a dramatic relative reduction of detectable IAA in the Col-0 ecotype. If flavonols directly inhibit IAA synthesis, *tt4* should result in increased IAA in Col-0. Neither was observed.

This data therefore does not support the hypothesis that flavonols directly inhibit auxin synthesis. Similarly, the data do not support the idea that flavonols affect IAA levels via reducing auxin transport – IAA levels in *tt4* Col-0 would be expected to increase in the absence of flavonols, but they remain unaffected. The same arguments apply to all flavonoids, and their derivatives since these are completely eliminated in the *tt4* mutants.

6.3.2 Analysing auxin response levels in unfertilised ovules

The work reported here sought a better understanding about the regulation of flavonoid genes, auxin transport, and the signalling mechanisms between the seed coat, embryo and

endosperm development. The comparison between auxin levels in unfertilised ovules and fertilised seeds is consistent with finding from Aloni *et al.* (2006) who reported elevated auxin levels in fertilised seeds, and suggested that this might be a result of both *de novo* auxin synthesis, and auxin accumulation from both gametes. This indicates that auxin has a potentially important role in the development of the seeds (Locascio *et al.*, 2014; Robert *et al.*, 2015).

6.3.3 Analysing auxin response levels in integuments seeds resulting from diploid and interploidy crosses between maternal FBP mutants and paternal Col2x and Col4x lines, respectively

6.3.3.1 Quantitative analysis of auxin response levels

Following pollination, immature seeds (3 to 9 DAP) (in diploid (DR5::GFP/FBP mutants (2x) X Col2x) and tetraploid (DR5::GFP/FBP mutants (2x) X Col4x) crosses) had a higher level of GFP signal in the funiculus, indicating that this tissue might act as a) a site of auxin synthesis, b) a sink for free auxin, and/or c) a main auxin transport tissue to the seed (Khan *et al.*, 2015). These results were again in accordance to findings in the literature about the inconclusive role of funiculus in auxin transport/synthesis (Aloni *et al.*, 2006; Khan *et al.*, 2015).

Furthermore, DR5::GFP/*ban*XCol2x, DR5::GFP/*ban*XCol4x, DR5::GFP/Col2xXCol2x and DR5::GFP/Col2xXCol4x seeds showed similar auxin increase patterns but different auxin response levels, where DR5::GFP/Col2xXCol4x had considerably higher auxin response at 7 DAP, whilst DR5::GFP/*ban* XCol4x had higher auxin levels at 9 DAP.

Moreover, as discussed in Chapters 3 and 4, *ban* had higher levels of anthocyanin, and in Chapter 5 that anthocyanin could have a role in the polar auxin transport to the integument layers, where *BAN* is expressed (Debeaujon *et al.*, 2003). Collectively, this could explain why DR5::GFP/*ban*XCol2x, DR5::GFP/*ban*XCol4x crosses had higher auxin levels than DR5::GFP/Col2xXCol2x, DR5::GFP/Col2xXCol4x crosses at 9DAP. In contrast, the fact that DR5::GFP/Col2xXCol4x seed had more auxin levels than DR5::GFP/*ban*XCol4x seed at 7DAP could indicate that anthocyanin did accumulate sufficiently to cause a shift in the levels, and that PAs, could have a role in the auxin transport at 7DAP; however, this statement tenuous due to the indirect evidence collected from results at 7DAP and the same origin of anthocyanin and PAs (cyanidin). Hence, more evidence is required to give this statement firmer support, such as analysis of GFP

levels in DR5::GFP/*anl1* lines. Auxin levels increased between diploid and tetraploid crosses, DR5::GFP/Col2xXCol2x, DR5::GFP/*ban*XCol2x, DR5::GFP/Col2xXCol4x and DR5::GFP/*ban*XCol4x seeds, which could be due to a response to mechanical stress (Nakayama *et al.*, 2012) that would be observed following the continuous enlargement of the endosperm against the integuments in the tetraploid cross (Scott *et al.*, 1998). In fact, the flux of auxin to the stressed tissue and the activity of the PIN1 protein (auxin efflux carrier) have been shown to augment as the result of mechanical stress on the tissues (Nakayama *et al.*, 2012), and several studies point out the important role of the flavonols in auxin transport mechanisms (Santelia *et al.*, 2008; Murphy *et al.*, 2002; Yin *et al.*, 2014). Moreover, this auxin increase could reflect a rise in paternally-derived growth signals from the endosperm or embryo to the maternally-derived tissues of the seed coat, which is consistent with the genome dosage hypothesis of parental conflict (Haig and Westoby, 1989). Both these explanations would lead to either a) stretching the cells in the endothelial layer and integuments as a way of relaxing the boundary provided by these tissues or b) increasing the rate of cell division (Garcia *et al.*, 2005; Haughn and Chaudhury, 2005; Schruff *et al.*, 2006). The seed coats of DR5::GFP/*fls*XCol2x, DR5::GFP/*ttg2*XCol2x, DR5::GFP/*fls*XCol4x and DR5::GFP/*ttg2*XCol4x crosses had a severely reduced amount of GFP signal.

Interestingly, Santelia *et al.* (2008) point out that the dehydrogenation of dihydroquercitin and dihydrokaempferol is catalysed by flavonol synthase (FLS) to quercetin and kaempferol, which are flavonols that are thought to play a role in the promotion of polar auxin transport (PAT). Therefore, it is not surprising that removing the documented modulators of PAT produces a phenotype that is radically different in auxin accumulation to the wild-type.

Conversely, as TTG2 plays a role in the regulation of the PA-specific steps of the FBP, *ttg2* mutant encodes WRKY transcription factor that is defective in both PA accumulation in vacuoles and PA synthesis, which would lead to an increase in anthocyanin levels (Johnson *et al.*, 2002; Debeaujon *et al.*, 2003). Therefore, the initial deduction would indicate similar results to that of *ban*.

However, there was a surprisingly low accumulation of auxin in seed coats with DR5::GFP/*ttg2*XCol2x, DR5::GFP/*ttg2*XCol4x seeds as compared to that of (WT) DR5::GFP/Col2xXCol2x, DR5::GFP/Col2xXCol4x and DR5::GFP/*ban*XCol2x, DR5::GFP/*ban*XCol4x. This could be taken as further evidence for the claims stated in

Chapter 5 about the role of TTG2 in the biosynthesis of PAs and anthocyanins, and hence auxin transport, as suggested earlier when discussing the *ban* data. Hence, better insights into these claims will require further investigation of auxin accumulation in the funiculus of *ttg2*, and around the specific role of this tissue in auxin synthesis and transport.

The mutation in *TT4* blocks the FBP at early steps, leading to little or no accumulation of the flavonoids end products in the vacuole. As shown in 6.2.1.2, DR5::GFP/*tt4*XCol2x, DR5::GFP/*tt4*XCol4x are associated with the relatively low level of auxin accumulation in the seed coat layers. In fact, auxin levels seem to be higher in the seeds resulting from the tetraploid cross (DR5::GFP/*tt4*XCol4x) at 3 and 5 DAP; whilst auxin levels were higher in the diploid cross (DR5::GFP/*tt4*XCol2x) at 7 and 9 DAP. These puzzling results, could be explained by proposing that as *tt4* is a knock out mutant, and that in the diploid cross (DR5::GFP/*tt4*XCol2x), the seed senses the lower levels in flavonoids at 3 and 5 DAP, and responds to this stress by increasing the auxin synthesis at 7 and 9 DAP, in the integument layers. However, this statement should be treated as a preliminary deduction to the results; further data is required such as auxin levels in the funiculus tissues and localisation and expression studies of auxin transport proteins, coupled with auxin quantification studies using 35S::DII-VENUS (see 6.5 below).

On the other hand, the results from seeds with maternal DR5::GFP/*tt4* and Col4x pollen paternal showed that auxin levels increased between 3 to 5 DAP and then decreased in the following days. Taking into consideration data from L*er* concerning the rescue nature of *tt4* seeds, this could be explained by auxin –the cellularisation factor- decreasing as a consequence of reduced flavonoids in tetraploid crosses.

6.3.3.2 Qualitative analysis of auxin response levels

Regarding the localisation of the DR5::GFP signal, WT and *ban* seeds had a clearer signal on the cell borders, more so in the tetraploid cross (DR5::GFP/Col2xXCol4x and DR5::GFP/*ban*XCol4x) than in the diploid cross (DR5::GFP/Col2xXCol2x and DR5::GFP/*ban*XCol2x).

However, GFP signal in DR5::GFP/*tt4*XCol2x and DR5::GFP/*tt4*XCol4x was more focused towards the inside of the cell. Could TT4 and the subsequent flavonoid control the position of the ER in the cell? The answer lies in the possibility that the stress induced by a decrease in the amount of flavonoids in DR5::GFP/*tt4*XCol2x and DR5::GFP/*tt4*XCol4x seeds leads to the formation of ER bodies, rather than changing the

location of ER organelles (Matsushima *et al.*, 2003; Yamada *et al.*, 2009).
Moreover, there fact that the ER-targeted DR5::GFP signal in DR5::GFP/Col2xXCol2, DR5::GFP/*ban*XCol2x, DR5::GFP/Col2xXCol4x and DR5::GFP/*ban*XCol4x seeds was localised to the cell borders could indicate that the ER networks are not established yet, and that the DR5::GFP is getting transported to the apoplast, which is the other auxin sink in the cell, where it gets expressed; hence the high signal in the cell borders.

6.3.4 The study of auxin response in the endosperm

These results, in addition to reports that the endosperm is the source of phytohormones (Lopes and Larkins, 1993), lead us to consider studying the auxin response in the endosperm. However, and as the DR5::GFP line used was ER-targeted (Benkova *et al.*, 2003; Friml *et al.*, 2003), it was not possible to visualise the auxin response in the endosperm using this reporter -within the time limits of this project-, especially since the cellularisation of endosperm in seeds involved in crosses with paternal Col4x show severe delay or does not occur (Day *et al.*, 2008; Le *et al.*, 2010).

Away from the experimental limitations regarding studying auxin response in the endosperm, it is worth noting that Day *et al.* (2008) performed a transcriptomic analysis at 4DAP, which implied that endosperm sensitivity to auxin was low at this time point and that, in line with cellularisation, at 5-6DAP, one would expect an increase in sensitivity. Nevertheless, the BAR *Arabidopsis* eFP database for the expression levels of ARF1 and TIR1 (essential components of the auxin signalling machinery) showed that even after 4DAP these genes are expressed at low levels (Winter *et al.*, 2007). Therefore, it seems that the absence of endosperm DR5::GFP signal could be also dampened at another level, due to auxin sensitivity, and thus, low auxin response.

6.3.5 Analysing the relation between vacuoles and flavonoids, through tt mutants

The results above point to a possible role for flavonoids in auxin transport, synthesis or response mediated via the vacuole. Analysis of the endothelium layer of the immature seed coat in DR5::GFP mutant tetraploid crosses showed that seeds resulting from interploidy crosses with maternal *tt4* had lytic vacuoles (Figure 6.10.B), whereas the other seeds had storage vacuoles (Figure 6.10). Biogenesis of vacuoles in plants involves secretory pathway compartments, leading to three types of vacuoles (Figure 6.11): Storage Vacuole (SV, salmon colour), Lytic Vacuoles (LV, light blue colour) and hybrid vacuoles (Neuhaus and Martinoia, 2011).

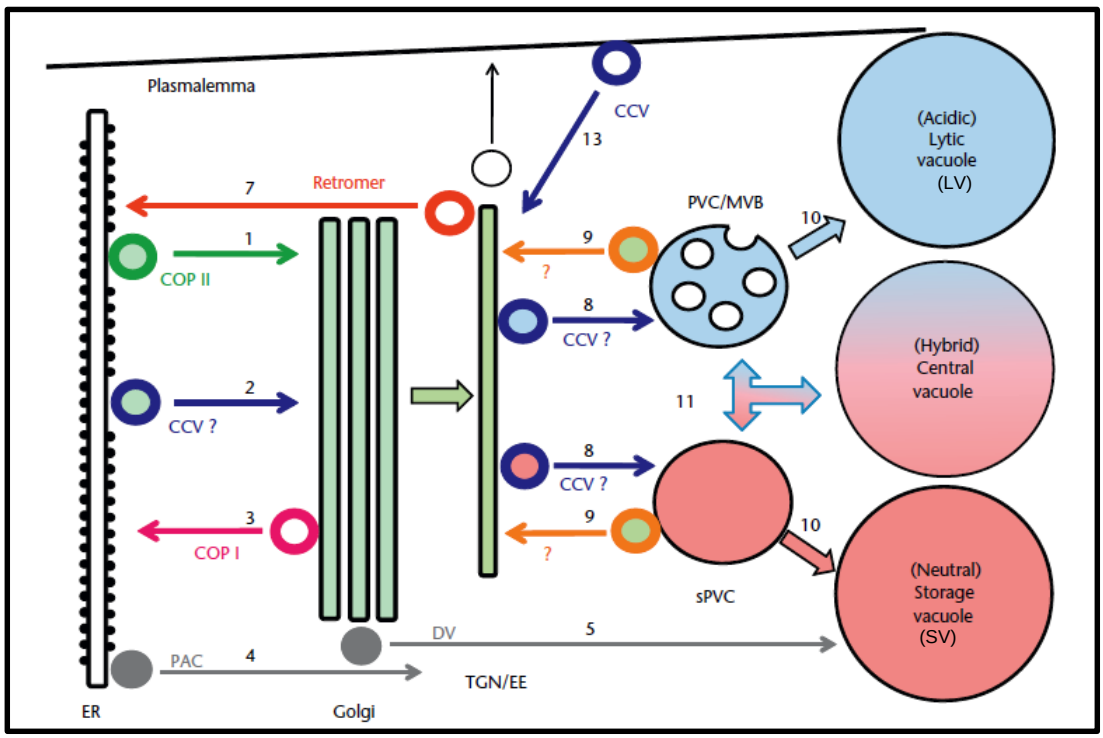


Figure 6.11: Vacuolar secretory pathway. Please note that, in accordance with the text above, the only relevant parts of this figure are steps 10 and 11. Neuhaus and Martinoia (2011) comment that the transport of storage pre-vacuole component (**sPVC**) (step 10) or multivesicular body (**MVB**) (step 11) to vacuoles by direct fusion, after the sPVC or MVB maturation process is completed, resulting a storage vacuole (SV) and lytic vacuole (LV), respectively. Storage vacuole is characterised by a large vacuole in all DR5::GFP/FBP mutantsXCol4x except DR5::GFP/*tt4*XCol4x that has a lytic vacuole. **Abbreviation: ER:** endoplasmic reticulum; **TGN/EE:** Trans-Golgi network/early endosome; **PVC/MVB:** pre-vacuolar compartment/multivesicular body; **PAC:** precursor-accumulating vesicles; **DV:** dense vesicles (Adapted from Neuhaus and Matinoia, 2011).

These observations suggest that TT4 and/or its subsequent flavonoids and regulatory elements (including FLS, BAN and TTG2) might either a) regulate the formation of storage vacuole, or b) regulate other molecules that in turn control the formation storage

vacuole. Additionally, the low levels of flavonoids produced in FBP mutants may not trigger a signal required to trigger the formation of storage vacuoles to store the flavonoids at normal control levels. It is worth noting that the results from this histology experiments described here were are consistent with Debeaujon *et al.* (2003) and Kitamura *et al.* (2010) who reported that *tt4* immature seeds lacked TB signals in endothelial cells because of the reduced flavonoid.

6.4 Conclusions

The purpose of this study was to investigate the localisation of auxin using the DR5::GFP auxin-response reporter as development progressed. The results of FBP mutants crosses, broadly agree with other studies showing that flavonoids have interplay with auxin transport. Moreover, the data provides good evidence that auxin movement in the integument layers is regulated by flavonoids. Thus, it may be the case that auxin creates the endosperm cellularisation signal (Dilkes *et al.*, 2008; Scott *et al.*, 2013; Doughty *et al.*, 2014); however, it is essential to study how that occurs by using auxin reporters rather than auxin response reporter alone.

Moreover, during seed development, auxin accumulates in the integuments progressively, which leads to an increase in post-fertilisation growth and cell division. Thus, it could be suggested that a higher level of auxin signal in the integuments of 2xX4x crosses than 2xX2x crosses, would accumulate, which might indicate that the increase in genome dosage gave rise to an increase in paternally derived signals.

As shown in previous studies, flavonoids were non-essential regulators of polar auxin transportation (Peer *et al.*, 2011). Nevertheless, this regulation was limited to the seed coat tissues, and the variation among mutants was massive.

6.5 Limitations and Future work

Auxin response studies gave inconclusive results. This could understand more by using defensin-like proteins specific to synergid and central cells in auxin biosynthesis pathway (*DD25::IaaH*), not auxin response reporter, to study the auxin accumulation in the endosperm and the funiculus. Studying auxin distribution and transport within these two

tissues was shown to be of particular importance to understand the effect of auxin on flavonoids and/or vice versa.

Additionally, because of the limitations of computer software, it proved impossible to capture or process Z-stacks of some of the largest seeds (i.e., 9DAP). For this reason, any future studies that use these methods of collecting data are recommended only to attempt the Z-stack capture of smaller or thinner organs and to reduce the number of steps by increasing Z-stack step size.

The development of a tissue-specific promoter-driven knockouts or even RNAi knockdown for auxin transporters in the endothelial layer would be useful for a future experiment that could discriminate a role for auxin as the maternal cellularisation signal. If one assumes that these active transport mechanisms are necessary for auxin to enter the endosperm, then a deliberate inhibition of auxin transport into this tissue could be achieved by this experiment. If the endosperm develops normally, modifications need to be made in the model of how auxin signals in the endosperm. This experiment would be far better in supporting or rejecting the hypothesis as it provides evidence, which is superior to just reporting the presence of auxin.

The IAA levels data (Figure 6.2) was inconsistence might be due to the selection of mutants and the stage of the seeds (5 DAP). Therefore, it would be better to cover different stages of development of FBP mutants (full knockouts).

Having histological sections (Figure 6.10) of a range of FBP mutants X Col4x crosses; control cross Col2x X Col4x in the Col-0 ecotype; and not the WT cross Col2x X Col2x was a limitation of the study, thus it would have been useful to have WT cross.

Chapter 7: General discussion and conclusion

7.1 Context for the investigation of the Flavonoid Biosynthesis Pathway and its role in modulating seed development following interploidy crosses

There is a projected global population increase to around 10 billion by 2050; therefore, the need to increase food production is of great current significance (FAO, 2009; Doughty *et al.*, 2014). Seeds constitute about 68% of the world's food supply and are thus a vital source of nutrition (Li and Berger, 2012). Seeds are the products of sexual reproduction in plants via double fertilisation and are made up of three components: a) the seed coat (testa), b) the embryo, and c) the endosperm (Gehring *et al.*, 2004). The development of the seed coat is determined by the maternal genome while the endosperm and the embryo develop under the control of both parental genomes (Li and Berger, 2012). The embryo, endosperm and integuments need to synchronise their growth during seed development. Studies have suggested that the interaction between the endosperm and integuments during growth is an important factor in the determination of seed size (Garcia *et al.*, 2005). Thus, the aberrant behaviour of the zygotic (including the endosperm) or maternal tissues has the potential to impact on seed size; this can result from unbalancing endosperm development as can occur following interspecific and interploidy hybridisation (Scott *et al.*, 1998). There is evidence that maternal modifier genes, such as regulatory or structural genes of the flavonoid biosynthesis pathway (FBP), can re-impose a normal (balanced) developmental programme on the endosperm and restore more-or-less normal viability (Dilkes *et al.*, 2008; Scott *et al.*, 2013). Moreover, there is some indirect evidence that the phytohormone auxin may act as a maternal endosperm cellularisation signal, and that could be modulated by flavonoids (Lopes and Larkins, 1993; Peer *et al.*, 2001; Thompson *et al.*, 2010).

Thus, taking all these points into consideration (please see Chapter 1: section 1.8 for more details), the purpose of this research was to: a) investigate the role of the FBP in regulating seed development, particularly with respect to the timing of endosperm cellularisation, and how that would affect the ability of some FBP mutations to rescue paternal Col4x-induced seed lethality, b) determine the FBP intermediates responsible for regulating the Col4x-killer effect, c) verify the FBP intermediates responsible for regulating the Col4x-killer impact, and d) investigate a potential role for auxin as a maternal endosperm cellularisation signal and its relationship to the FBP.

7.2. Linking seed lethality in interploidy crosses to the FBP

The need to create larger seeds and the reports linking polyploid genomes to increased seed size/weight has led researchers to perform intraspecies/interploidy crosses (Scott *et al.*, 1998; Dilkes *et al.*, 2008). For example, the crosses Ws2x X Ws4x and L*er*2x X L*er*4x produce heavier seeds (Bolbol, 2010) however, Col2x X Col4x crosses lead to a high frequency of seed lethality (shrivelled seeds). Ws2x and L*er*2x crosses to Col4x pollen parents also produce seeds with a lethal –shrivelled- phenotype, although the percentage of shrivelled seeds is much higher in the Col2x X Col4x cross (Dilkes *et al.*, 2008; Bolbol, 2010). Thus, the paternal effect of Col4x started to be recognised as a particularly potent inducer of seed lethality in crosses to 2x seed parents, a phenomenon known as "triploid" block, due to the fact that resulting seeds would have a lethal 3x genome, rather than the viable 2x genome.

Importantly it has recently been found that loss-of-function of some or all of the FBP within the endothelial layer is responsible for seed rescue in crosses with paternal Col4x; this has led to an alternative to the 'integument size restriction model'[7] being put forward to explain the rescue effect (Garcia *et al.*, 2005; Scott *et al.*, 2013). The alternative 'cellularisation-signalling model' comes out of investigations involving mutation of the FBP and it hypothesises that increased transport of a cellularising factor between the maternal integuments and the endosperm causes precociousness in endosperm cellularisation. Moreover, it has been reported that the majority of transparent testa (*tt*) mutants (encoding proteins involved in FBP that when knocked out cause lighter seed pigmentation) in the L*er* ecotype rescue Col4x induced lethality in seeds resulting from interploidy crosses (Scott, pers. comm.).

7.3. Are the levels of flavonoids responsible for inducing/ reducing the triploid block?

Several steps of the FBP were chosen to be disrupted using a reverse genetic approach. The targeted enzymes/proteins were responsible for a) generating the bulk of flavonoids, due to their role in the early steps of biosynthesis (TT4, TT5, TT6, TT7, TT11/18), b) flavonol biosynthesis (FLS), c) anthocyanin biosynthesis (ANL1), d) proanthocyanidins

[7] The integument cells in *ttg2* mutants are less elongated resulting in precocious –early- endosperm cellularisation (Garcia *et al.*, 2005). It has been suggested that no clear relationship between viability and embryo sac size. The embryo sac size in *ttg2-1* X Col4x seed is smaller than L*er* 2x X Col4x while embryo sac size in *ttg2-3* X Col4x was an identical to Col2x X Col4x embryo sac size. Therefore, this indicating *ttg2* work through another mechanism (Dilkes *et al.*, 2008).

(PAs) biosynthesis (BAN) and their oxidation (TT10), e) transport of anthocyanin and PAs into the vacuole (TT12, TT19/14 and AHA10), and f) regulating the transcription of several key enzymes in the FBP (TTG2 transcription factor).
This was coupled to a double mutant analysis to specifically a) stop the accumulation of anthocyanin and PAs (*anl1* and *ban*), b) stop the accumulation of anthocyanin and its transport to the vacuole (*anl1* and *tt12*) but potentially permit accumulation of the PA precursor epicatechin in the cytosol and c) stop the accumulation of PAs and their transport to the vacuole (*ban* and *tt12*) but potentially permit accumulation of anthocyanins in the cytosol.
It should be noted that not all the T-DNA insertion mutations in genes of the FBP led to complete knockouts. Based on several lines of evidence (see Chapter 3: section 3.2.4) a number were considered at very least as functional 'knockdowns' (*tt4, tt5, tt7, tt3, fls, ban, aha10* and *ttg2*). However, phenotypic analysis based on mass spectrometry and seed colour data showed that there was variation in the flavonoid levels/effects between WT and these 'knockdown' mutant lines.

The main criteria for assessing whether components of the FBP have any effect on ameliorating the triploid block caused by Col4x, is to check if any FBP mutation in this pathway leads to 'rescue' phenotypes. These rescue phenotypes can be divided into two categories of evidence: a) the first category, (which is within the scope of this project), was to generate data on the weight and shape (shrivelled vs. plumbed) of the seeds; b) the second category (which would be considered as a continuity of this this project) would be to study the germination percentage of the seeds generated from these interploidy crosses. The main drive of this project was to cover as many mutants of the FBP as possible and elucidate their effects on seed development; this focus and time constraints of the project meant the move to the second line of investigation (germination studies) was not possible.

In the early stages of the project, the effect of various EMS mutations of the FBP in maternal L*er*, Ws, and Col-0 lines crossed with paternal Col4x were studied. Data obtained from this analysis supported the hypothesis that the FBP does influence seed development probably at the level of endosperm cellularisation. It also indicated that the two measures 'weight' and % of 'plump' seed were good indicators of 'rescue' - for example, seeds generated from maternal *ttg2* had rescue phenotypes in L*er*, whereas

seeds from maternal *tt12* had rescue phenotype in Ws. However, the fact that the controls (seeds resulting from crosses between maternal Col2x, L*er*, and WS, and paternal Col4x) produced different results highlighted the high degree of ecotypic variation for Col4x-induced seed lethality. Such variation would complicate any meaningful dissection of the role of the FBP in the context of this study. This highlighted the urgent need for research using a single ecotype (Col-0).

The results from the Col-0 FBP mutant study that several, though not all, mutants were capable of rescuing Col4x-induced seed lethality. Seed weight and the percentage of plump/burst seeds were consistent with one another as indicators of 'rescue'. *tt4*, *tt6*, *tt3*, *tt12*, *aha10*, and *ttg2* were found to be the most effective at inducing a degree of rescue. *tt4* and *tt6* are enzymatic steps operating early in the FBP (see Figure 7.1) that would potentially block the production of all main classes of flavonoids, with *tt3* acting later (flavonol biosynthesis would not be blocked in *tt3*). Interestingly both *tt12* and *aha10*, which act at the end of the FBP and affect the import of flavonoids into the vacuole, had very strong rescue phenotypes. Both *tt12* and *aha10* are reported to have marked vacuolar morphological defects and in addition fail to accumulate PAs (Baxter *et al.*, 2005; Pourcel *et al.*, 2005; Zhao *et al.*, 2010).

In the more extensive second part of this study where another body of data was generated for *fls-1* (Col-0) the phenotype was less severe but nevertheless *fls-1* (Col-0) still failed to rescue Col4x induced lethality. This is most likely attributed to the effect the *fls-1* (Col-0) mutation has on the FBP; with the loss of the flavonol producing branch of the pathway, the flux of metabolites down the FBP will increase. However, the loss of the flavonol does not occurs because of the FLS redundancy (Appelhagen *et al.*, 2014), perhaps resulting in a higher accumulation of flavonols and undefined flavonoid compounds that delay affect endosperm cellularisation and hence increase lethality in the seed (Stracke *et al.*, 2009) (Figure 7.1).

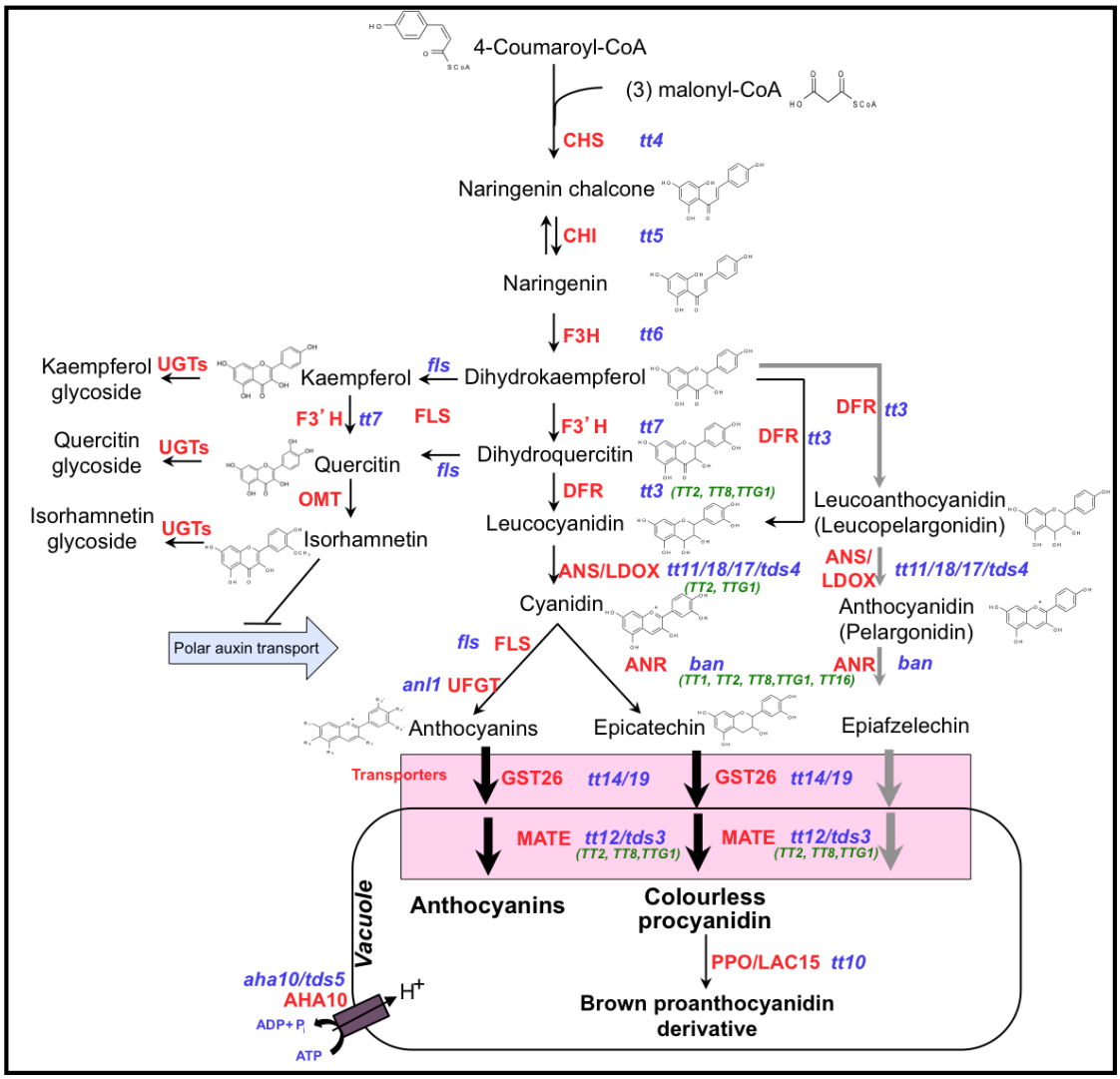


Figure 7.1: The updated flavonoid biosynthesis pathway in *A. thaliana* seeds. The *tt* mutants are shown in blue. The pink box shows transporters into the vacuole. The vacuole is indicated by the black box. Moreover, grey arrows illustrate an alternative routes found in the *tt7* mutant. Green fonts display transcription factors. The white box indicates the vacuole. Enzymes are shown in a red bold font and their names are: **CHS:** Chalcone synthase; **CHI:** Chalcone isomerase**; F3H:** Flavanone-3-hydroxylase**; F3'H:** Flavonoid-3'-hydroxylase**; FLS:** Flavonol synthase**; OMT:** *O*-methyltransferase**; UGTs:** UDP-dependent glycosyltransferase; **DFR:** Dihydroflavonol 4- reductase**; ANS/LDOX:** Anthocyanidin synthase/Leucoanthocyanidin dioxygenase**; UGFT:** UDP-glucose: flavonoid 3-O-glucosyltransferase**; ANR:** Anthocyanidin reductase**; GST:** Glutathione S-transferase**; MATE:** MATE secondary transporter; **AHA10:** Autoinhibited H^+-ATPase 10; **PPO/LAC15:** Polyphenol oxidase/ Laccase-15 (modified from Winkel-Shirley, 2001; Debeaujon *et al.*, 2003; Baxter *et al.*, 2005; Pourcel *et al.*, 2005; Lepiniec *et al.*, 2006; Kleindt *et al.*, 2010; Gou *et al.*, 2011; Appelhagen *et al.*, 2014; Mizzotti *et al.*, 2014; Ishihara *et al.*, 2016).

LC-ESI- QQQ-MS/MS analysis of immature *tt4* and *ttg2* seeds (5DAP), revealed them to have lower levels of flavonols, anthocyanin A1 and soluble procyanidin, thus indicating a strong relationship between lower levels of FBP products and rescue of seeds from the triploid block.

Interestingly seeds resulting from maternal *anl1* (having less anthocyanin and potentially more PAs) –as confirmed by Kubo *et al.* (2007)- had more extreme seed lethality than the control Col2x X Col4x cross. Furthermore, Kubo *et al.*, (2007) stated that a possible explanation of the WT-like *anl1* seed colour phenotype -although having a decreased anthocyanin level- were faults in a) the regulatory gene of the anthocyanin pathway, or b) a synthetic gene in the late step of anthocyanin biosynthesis in *A. thaliana.* Whereas seeds resulting from maternal *ban* (less PAs and more anthocyanin) (Routaboul *et al.*, 2006) did not show significant variation in rescue phenotypes from control cross – it should be noted that the *ban* T-DNA line was a knockdown which may explain this phenotype. It was the double mutant data, however, that gave an insight into a potential role of "cytoplasmic" anthocyanin as one of several possible 'rescue' factors. Seeds produced from crosses with the *ban/tt12* double mutant were significantly heavier than those from either *ban* or *tt12* (Figure 4.6) indicating that more anthocyanin production, but also less transport to the vacuole resulted in a strong 'rescue' phenotype (Zhao and Dixon, 2010; Shi and Xie, 2014; Chanoca *et al.*, 2015). However it is difficult to explain a specific role for anthocyanin when one considers that *tt4* mutants that lack anthocyanin have a strong rescue phenotype though it is quite possible that a number of independent mechanisms operate in 'rescue' of Col4x-induved seed lethality.

Thus, the results derived from the study of mutants of the FBP provide valuable insights into potential mechanisms for the alleviation of paternal Col4x-induced seed lethality in *A. thaliana*. Three possibly interlinked rescue mechanisms are proposed:

1) Reduced biosynthesis of all main flavonoid groups in the endothelium influences developmental signalling between maternal and zygotic tissues - based on data obtained from *tt4* and *ttg2* seed parents.
2) Reduced/altered levels of FBP products including and downstream of cyanidin. In particular, a loss of PAs is consistently associated with a 'rescue' phenotype – based on data obtained from *tt11/18, tt12, aha10, anl1*, and *ban/tt12* seed parents.

3) Aberrant endothelial vacuolar morphology. Loss of a normal single central vacuole that accumulates PAs is a striking feature of *tt12*, *aha10* and *tt11/18* mutants that instead feature multiple small vesicle-like structures in the cytoplasm. Loss of the main central vacuole goes hand-in-hand with loss of PA accumulation and thus loss of the vacuole itself, rather than PAs, may be the primary cause of rescue from Col4x-induced lethality (Figure 7.2).

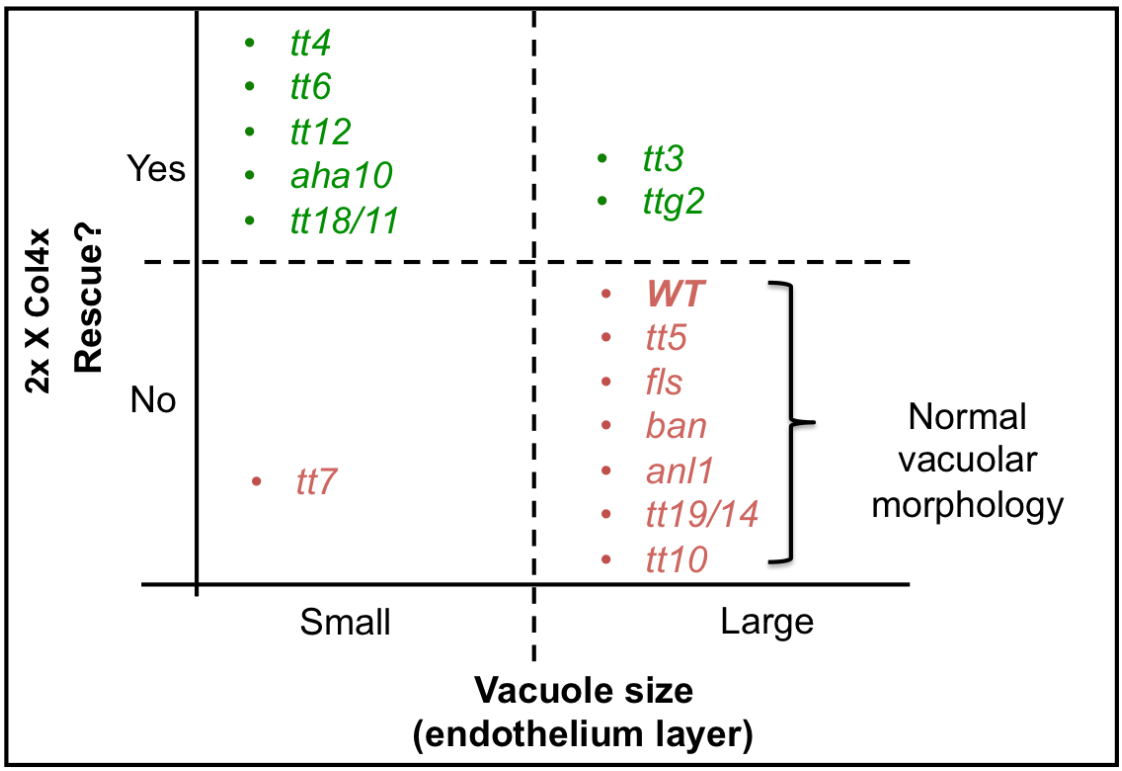


Figure 7.2: shows the correlation between vacuole sizes in the endothelium layer and rescue of Col4x in seeds resulting from FBP mutants X Col4x. Rescue mutants of Col4x induced seed lethality were shown in green font had small vacuole size except *tt3* and *ttg2* mutants. However, lethal crosses were shown in red front had large vacuole which is normal vacuolar morphology except *tt7* mutant.

A role for auxin in modulating 'rescue' in FBP mutants was also sought in this study. Interestingly, LC-ESI- QQQ-MS/MS data showed that developing seeds from the FBP mutants studied contained different levels of auxin, indicating that either auxin has a role in the FBP or more likely that products within the FBP can influence the levels of auxin. However, this data is preliminary and should be treated with caution as samples were whole immature seeds (5 DAP) that due to the difficulties of dissection had remnants of the funiculus attached (which contains significant accumulations of auxin – personal observation). Hence, localisation of auxin was performed in seeds resulting from crosses between maternal FBP mutants carrying the DR5::GFP auxin reporter construct and

Col4x pollen parents. These studies revealed that auxin levels in the seed coat did indeed vary between the different mutant backgrounds. Moreover, results from seeds generated from maternal *ban* (*ban* fails to rescue Col4x-induced seed lethality), showed that auxin levels were higher than controls in the seed coat, indicating a possible relation between cyanidin derived flavonoids and auxin – though it remains possible that pathway regulation is altered in FBP mutants and levels other flavonoids earlier in the pathway could be altered. Furthermore, seeds resulting from maternal *tt4* and *ttg2* (which were good rescuers of Col4x-induced seed lethality) had lower auxin levels in the seed coat, thereby, further indicating a relationship between auxin and flavonoids downstream of TT4. A number of studies have provided evidence that certain aglycones flavonols have a role in auxin transport (Jacobs and Rubery, 1988; Peer *et al.*, 2001; Peer *et al.*, 2004; Besseau *et al.*, 2007; Santelia *et al.*, 2008; Buer *et al.*, 2013), however a clear correlation could not be established in this study between levels of flavonols in FBP mutants and the 'rescue' phenotype. Due to limitations in the mechanism by which the DR5::GFP construct 'reports' auxin levels it was not possible to monitor the levels of auxin in endosperm (Kohler, pers. comm.). This was unfortunate given that cellularisation of endosperm tissue is of central interest to this project, though the data that was obtained does strongly indicate that auxin transport is likely affected in some FBP mutant lines. Whether altered auxin distribution is a factor in the alleviation of Col4x-induced seed lethality remains to be determined. It is suggested that future studies could utilise a more effective auxin line such as *DD25::IaaH*, and could potentially included in combination with FBP mutants polar auxin transport mutants, in order to provide a better insight into auxin localisation and effect its effect on endosperm development.

In conclusion, dissecting the specific role(s) played by the FBP in regulating seed development has proved particularly challenging. In part, this has been due to the severity of the triploid block in the Col-0 ecotype where 'rescue' phenotypes were more subtle and difficult to evaluate compared to other ecotypes such as L*er* and Ws. However, promising leads have been uncovered in this study, especially regarding the roles of cyanidin derived flavonoids (anthocyanins and PAs), auxin, and altered vacuolar biogenesis in influencing seed rescue from Col4x-induced lethality.

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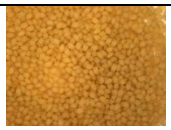
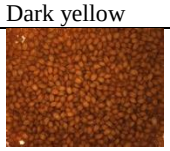
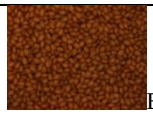
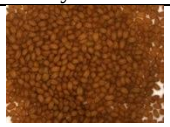
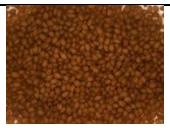
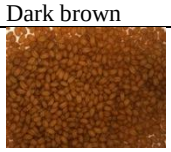
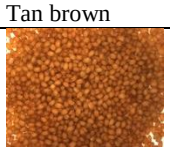
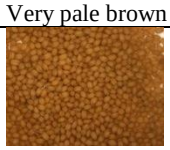
Appendixes

Appendix A: FBP mutants phenotypes in *A. thaliana* ecotypes

Table 1: Characterisation of FBP mutant's seed coat colour in *A. thaliana* ecotypes.

Flavonoid biosynthesis genes and regulatory gene	Seeds coat colour in Col-0 ecotype	Seed coat colour as reported in literature	References
tt4	 Yellow	 Pale yellow in L*er* and Col-0	Debeaujon *et al.*, 2000; Bowerman *et al.*,2012
tt5	 Pale tan brown	 Yellow in L*er* and Col-0	Debeaujon *et al.*, 2000; Bowerman *et al.*,2012
tt6	 Yellow	 Very pale brown in L*er* and Col-0	Debeaujon *et al.*, 2000; Bowerman *et al.*,2012
tt7	 Pale brown	 Very pale brown in L*er* and Col-0	Debeaujon *et al.*, 2000; Bowerman *et al.*,2012
tt3	 Dark yellow	Yellow in L*er*	Debeaujon *et al.*, 2000
fls1	 Brown (similar to WT colour)	 Brown in Col-0	Wisman *et al.*, 1998
tt11/tt18	 Dark yellow	 Pale brown in L*er*, Ws and Col-0	Debeaujon *et al.*, 2000; Abrahams *et al.*, 2003; Bowerman *et al.*,2012
ban	 Dark brown	 Grayish- brown in L*er* and Col-	Devic *et al.*, 1999; Xie *et al.*, 2003; Bowerman *et al.*,2012

anl1	 Dark brown	Brown in Col-0	Kubo *et al.*, 2007
tt14/tt19	 Tan brown	Pale/ dark brown (pale brown at harvest and get darker when storage) in L*er* and Col-0	Debeaujon *et al.*, 2000; Kitamura *et al.*, 2004
tt12	 Very pale brown	Pale brown in L*er* and Ws	Debeaujon *et al.*, 2000;
tt10	 Very Pale brown	Pale/ dark brown (pale brown at harvest and get darker when storage) in L*er* and Ws	Debeaujon *et al.*, 2000; Pourcel *et al.*, 2005
aha10	 Very pale brown	 Pale brown in Ws	Baxter *et al.*, 2005
ttg2	 Very pale brown	Yellow in Ws and L*er*	Johnson *et al.*, 2002; Debeaujon *et al.*, 2003

Appendix B: The data of FBP single and double mutants crossed by Col4x

Table 4.1: Seed cavity area ratio between FBP mutants and the control of crosses. All crosses were to Col4x pollen parents, and data is shown for seeds at 5 and 7 DAP.

tt mutants X Col4x	Average seed cavity area			
	5DAP	7 DAP	Ratio 5DAP	Ratio 7DAP
Col2x (10,10)	40026	50771		
anl1 (10,10)	40797	80717	1.01	1.58
ban (10,10)	45599	101176	1.13	1.99
tt12 (10,10)	43772	47249	1.09	0.93
anl1/ban (10,10)	47056	77185	1.17	1.52
ban/tt12 (10,10)	43100	119737	1.07	2.35
anl1/tt12 (10,10)	33054	81171	0.82	1.59

Table 4.2: Chalazal endosperm area ratio between FBP mutants and the control of crosses. All crosses were to Col4x pollen parents, and data is shown for seeds at 5 and 7 DAP.

tt mutants X Col4x	Average chalaza area			
	5 DAP	7 DAP	Ratio 5 DAP	Ratio 7 DAP
Col2x (10,10)	2645	6312		
anl1 (10,10)	764	5255	0.28	0.83
ban (10,10)	881	6430	0.33	1.01
tt12 (10,10)	2069	4274	0.78	0.67
anl1/ban (10,10)	1064	5343	0.40	0.84
ban/tt12 (10,10)	1875	12119	0.70	1.91
anl1/tt12 (10,10)	1526	4232	0.57	0.67

Table 4.3: Ratio between the number of nodules in seeds derived from FBP mutants and the Col 2x control crossed to Col4x pollen parents. Data is shown for 5 and 7 DAP.

tt mutants X Col4x	Average nodule number			
	5 DAP	7DAP	Ratio 5 DAP	Ratio 7 DAP
Col2x (10,10)	0.8	0.5		
anl1 (10,10)	0.7	1.9	0.87	3.8
ban (10,10)	1.9	2.4	2.37	4.8
tt12 (10,10)	1.3	1.2	1.62	2.4
anl1/ban (10,10)	1.3	3.7	1.62	7.4
ban/tt12 (10,10)	1.7	0.7	2.12	1.4
anl1/tt12 (10,10)	0.8	1.1	1	2.2

Table 4.4: Mean seed weight ratios about control seed weights (Col2x X Col4x). Data is for seeds resulting from crosses between FBP single and double mutants and Col4x pollen parents at 5 and 7 DAP.

tt mutants X Col4x	Average individual seed weight	Ratio seed weight
Col2x (10,517)	14	
anl1 (6,210)	6	0.42
ban (9,178)	14	1
tt12 (12,509)	21	1.5
anl1/ban (10,273)	16	1.14
ban/tt12 (6,227)	25	1.78
tt12/anl1 (5,167)	18	1.28

Table 4.5: Ratio between the number of 'Plump and Burst' seeds derived from FBP mutants and the Col 2x control crossed to Col4x pollen parents. Data is shown for 5 and 7 DAP.

tt mutants X Col4x	Average % 'plump and burst' seeds	Ratio 'plump and burst' seeds
Col2x (10,517)	6	
anl1 (6,210)	5	0.83
ban (9,178)	18	3
tt12 (12,509)	54	9
anl1/ban (10,273)	4	0.66
ban/tt12 (6,227)	27	4.5
tt12/anl1 (5,167)	17	2.8